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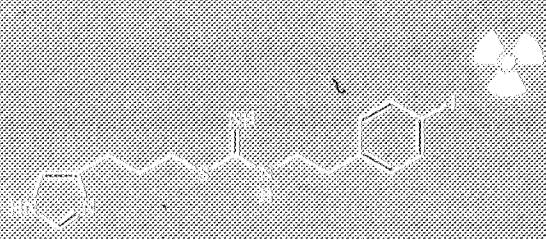

Frank P. Jansen

Histamine H_3-receptors in the central nervous system of rats and mice

Characteristics, distribution and function studied with [^{125}I]iodophenpropit

Ph.D.-thesis

Most of the research described in this thesis was performed at the Division of Molecular Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR), Department of Pharmacochemistry, Vrije Universiteit, De Boelelaan 1083, 1081HV, Amsterdam, The Netherlands.
Some of the investigations were carried out in close collaboration with other institutes. The work described in Chapter 6 was done in cooperation with the European Graduate School of Neuroscience, EURON, Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, The Netherlands. Part of the experiments presented in Chapter 7 were performed at the Division of Toxicology, Department of Pharmaceutical Biosciences, Uppsala Biomedical Center, Uppsala University, Uppsala, Sweden, and research described in Chapter 8 was performed at the Department of Medical Physics, School of Allied Health Sciences, Faculty of Medicine, Osaka University, Osaka, Japan.

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VRIJE UNIVERSITEIT

HISTAMINE H_3-RECEPTORS IN THE CENTRAL NERVOUS SYSTEM OF RATS AND MICE

Characteristics, distribution and function studied with [^{125}I]iodophenpropit

ACADEMISCH PROEFSCHRIFT

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Frank Pieter Jansen

geboren te Amsterdam

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Contents

· *Chapter 1* ·

Introduction

Contents

1. General introduction

The visualization in the early eighties of the histaminergic neuron system has provided new perspectives in histamine research. Histaminergic neurons are widely spread in the vertebrate and the invertebrate central nervous system (CNS), and constitute an important regulatory entity of brain activity (Wada *et al.*, 1991; Schwartz *et al.*, 1991; Schwartz *et al.*, 1995). Soon after the identification of the histaminergic system, the histamine H_3-receptor was discovered, mediating the feedback inhibition of neuronal histamine release and synthesis (Arrang *et al.*, 1983; Arrang *et al.*, 1987b). H_3-Receptors were additionally found to regulate the release of other neurotransmitters via presynaptic and postsynaptic mechanisms, both in the central nervous system and in peripheral tissues (Schwartz *et al.*, 1990; Timmerman, 1990; Schlicker *et al.*, 1994c). Hence, the H_3-receptor plays an essential role in the integration of histaminergic signal transmission in the CNS and in the peripheral nervous system (PNS). The understanding of the physiological and pathophysiological role of the H_3-receptor is considered to yield new therapeutic opportunities in CNS and PNS diseases (Arrang *et al.*, 1989; Schwartz *et al.*, 1990; Onodera *et al.*, 1994; Leurs *et al.*, 1995b; Alves-Rodrigues, 1996).
Progress in receptor pharmacology is largely dependent on the availability of potent and selective drugs. During the last ten years various H_3-receptor ligands belonging to distinct chemical classes have been found, including valuable tools for *in vitro* and *in vivo* studies (recent review: Leurs *et al.*, 1995b). Moreover, eight different H_3-receptor radioligands were developed. H_3-Receptor binding studies have substantially contributed to the current knowledge of characteristics, distribution and function of H_3-receptors.
The present chapter starts with a brief introduction on the functional role of H_3-receptors in the CNS and in peripheral tissues. Thereupon, H_3-receptor ligands will be reviewed, with emphasis on the different H_3-receptor radioligands and their application in H_3-receptor research.

1.1. Histaminergic neurons in the CNS: distribution and function

The role of histamine as a member of the monoaminergic transmitters in the invertebrate and mammalian CNS is now well established. The first evidence for the existence of histamine in nervous tissue was obtained at the end of the 1960s (Carlini & Green, 1963; Kataoka & De Robertis, 1967). After the development of a radioisotopic assay for the histamine synthesizing enzyme L-histidine decarboxylase it was shown that histamine is synthesized in histaminergic neurons (Taylor & Snyder, 1971; Baudry *et al.*, 1973). Also it was demonstrated that histamine is released from nervous tissue in a depolarization

and calcium dependent way (Atack & Carlsson, 1972; Taylor & Snyder, 1973; Verdiere *et al.*, 1975). Lesion studies provided preliminary information on the distribution of histaminergic neurons in rat brain (Garbarg *et al.*, 1974). Disruption of the medial forebrain bundle at the level of the lateral hypothalamic area resulted in a reduction of L-histidine decarboxylase activity and of histamine content in different forebrain areas indicating an ascending histaminergic pathway passing through the hypothalamus (Garbarg *et al.*, 1974). In the 1980s, the distribution of the histaminergic system in the rat brain was visualized immunocytochemically with antibodies against histamine conjugates (Wilcox & Seybold, 1982; Panula *et al.*, 1984; Panula & Airaksinen, 1991) and histidine decarboxylase (Watanabe *et al.*, 1983; Watanabe *et al.*, 1984; Tohyama *et al.*, 1991; Wouterlood & Steinbusch, 1991).

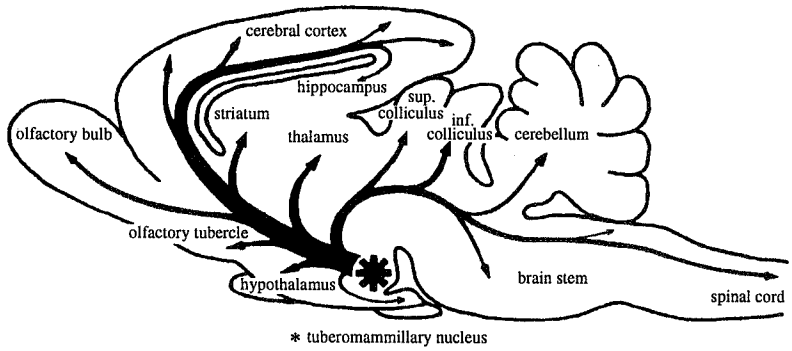


Figure 1. *Schematic presentation of the histaminergic system in rat brain. (Modified from Wada et al., 1991).*

Histaminergic neurons originate from five distinct cell groups (E1-E5) located in the tuberomammillary neuron complex of the posterior hypothalamus (see Figure 1). With respect to their efferents, the histaminergic perikarya can be regarded as a single neuronal group, projecting diffusely throughout the CNS in ascending and descending pathways (Wada *et al.*, 1991). The hypothalamus itself belongs to the most densely innervated areas, especially the suprachiasmatic, the supraoptic, the arcuate and the paraventricular nuclei. Ascending pathways project through the medial forebrain bundle and periventricularly to the olfactory bulb, most subcortical structures and the cerebral cortex. Of these areas a high innervation is found in the olfactory tubercle and various thalamic nuclei. Some histaminergic fibers also enter the median eminence terminating in the

pituitary (Steinbusch & Mulder, 1984; Inagaki *et al.*, 1988). Descending pathways project to various mesencephalic structures and to the spinal cord. Apart from neuronal elements, some histaminergic varicosities make contact with glial cells, small blood vessels and capillaries (Tohyama *et al.*, 1991). Also, long dendrites penetrate into the ependyma and make contact with the cerebrospinal fluid (Ericson *et al.*, 1987; Wada *et al.*, 1991). Electrophysiological studies indicated that histaminergic neurons fire spontaneously and regularly (Haas & Reiner, 1988). Most of the histaminergic fibers are unmyelinated and make relatively few synaptic contacts (Tohyama *et al.*, 1991).
Besides the rat brain, the histaminergic system has also been studied immunohistochemically in the CNS of other vertebrates (Onodera *et al.*, 1994) including the human brain (Panula *et al.*, 1990; Airaksinen *et al.*, 1991). In human brain, histaminergic neurons occupy a larger area of the hypothalamus and are about thirty times more abundant as compared to the rat (Airaksinen *et al.*, 1991). Despite species differences of the localization of the histaminergic perikarya, the tuberomammillary neuron complex can be regarded as one functional entity in all species examined (Airaksinen *et al.*, 1991; Panula & Airaksinen, 1991).

From studies with local and intracerebroventricular injections of histamine, ligands selective for histamine receptor subtypes (i.e. H_1, H_2, and H_3), and studies with the histidine decarboxylase inhibitor α-fluoromethylhistidine, several functions of the histaminergic system have been identified. Histamine has been assigned a 'wake amine'. Activation of the histaminergic system decreases sleep and increases wakefulness (for review see: Schwartz *et al.*, 1991; Onodera *et al.*, 1994) and locomotor activity (reviewed by Onodera *et al.*, 1994). Correlating to the 'wake' effects of histamine, the activity of histaminergic neurons in the brain displays a circadian rhythm, histamine levels being elevated during the active phase and decreased during the nonactive phase (Mochizuki *et al.*, 1992).
Several functions of the histaminergic system may originate from the hypothalamus which, as already mentioned, contains the histaminergic perikarya and has one of the highest densities of histaminergic terminals. A role of the hypothalamic histaminergic system in feeding, drinking, autonomic regulation (cardiovascular, respiratory and gastrointestinal modulation, thermoregulation), and pituitary hormone secretion has been indicated (reviewed by Onodera *et al.*, 1994). Histaminergic mechanisms may also be involved in learning and memory processes (Kamei *et al.*, 1993; Smith *et al.*, 1994; Meguro *et al.*, 1995; Miyazaki *et al.*, 1995; Blandina *et al.*, 1996; Sziklas *et al.*, 1996) and may play a modulatory role in epileptic seizures (Yokoyama *et al.*, 1992; Yokoyama & Iinuma, 1996). Moreover, the histaminergic system has been suggested to be affected

in schizophrenia (Prell *et al.*, 1995) and in neurodegenerative diseases like Alzheimer's disease (Airaksinen *et al.*, 1991; Nakamura *et al.*, 1993) and multiple system atrophy (Nakamura *et al.*, 1996).

1.2. The histamine H_3-receptor: the autoreceptor of histaminergic neurons

Differently from other monoaminergic neurotransmitters, for histamine and for its precursor histidine, no uptake mechanism selective for histaminergic neurons could be demonstrated (Arrang *et al.*, 1983; Smits *et al.*, 1988). Histamine is selectively synthesized in histaminergic neurons, due to the exclusive presence of L-histidine decarboxylase in these neurons. Consequently, histaminergic neurons can be selectively labelled with [^{3}H]-histamine *in vitro* by incubation of brain slices with [^{3}H]-histidine. Using this procedure, Arrang and coworkers (Arrang *et al.*, 1983) demonstrated that histamine is able to inhibit its own potassium-induced release (up to 60% of control) from rat cerebral cortex slices by activation of a histaminergic autoreceptor. Based on the deviating potencies of selective H_1- and H_2-receptor ligands in this assay, the effect of histamine was proposed to be mediated by a novel class of histamine receptors, the histamine H_3-receptor (Arrang *et al.*, 1983). A few years later, the existence of the histamine H_3-autoreceptor was further documented by the introduction of the first selective ligands to study histamine H_3-receptors i.e. the agonist (R)α-methylhistamine and the antagonist thioperamide (Arrang *et al.*, 1987a). Using an *in vitro* superfusion technique, H_3-receptor activation was also shown to inhibit neuronal histamine release induced by electrical stimulation (Van der Werf *et al.*, 1987). More recently, modulation of rat hypothalamic histamine release by activation of the H_3-autoreceptor has been evidenced *in vivo,* using cerebral microdialysis (Mochizuki *et al.*, 1991; Itoh *et al.*, 1992; Prast *et al.*, 1994; Chapter 8 of this thesis). H_3-Receptor mediated inhibition of histamine release was also observed in human cerebral cortex slices (Arrang *et al.*, 1988). As an autoreceptor, activation of histamine H_3-receptors was also shown to inhibit the synthesis of histamine (Arrang *et al.*, 1987b).

1.3. Regulation of histaminergic neuronal activity by heteroreceptors

Neuronal histamine release and synthesis were shown to be affected by different heteroreceptors. Depolarization induced [^{3}H]histamine release from rat cerebral cortex or hypothalamic slices *in vitro* was inhibited by activation of α_2-adrenoceptors (Hill & Straw, 1988; Gulat-Marnay *et al.*, 1989), M_1-muscarinic (Gulat-Marnay *et al.*, 1989; Ono *et al.*, 1992), κ-opioid (Gulat-Marnay *et al.*, 1990) and galanin receptors (Arrang

et al., 1991). Histamine release was increased by activation of μ-opioid receptors (striatal tissue, Itoh *et al.*, 1988) and of nicotinergic receptors (Ono *et al.*, 1992). Using microdialysis, neuronal histamine release was shown to be modulated *in vivo* by stimulation of α_2-adrenoceptors (Prast *et al.*, 1991; Laitinen *et al.*, 1995), NMDA receptors (Okakura *et al.*, 1992), IL-1β-receptors (Niimi *et al.*, 1994), μ-opioid receptors (Chikai *et al.*, 1994; Chikai & Saeki, 1995), $GABA_A$-receptors (Okakura-Mochizuki *et al.*, 1996) and $GABA_B$-receptors (Okakura-Mochizuki *et al.*, 1996).
Histamine turnover was found to be affected by μ-receptors (Itoh *et al.*, 1988), muscarinic receptors (Oishi *et al.*, 1990), nicotinergic receptors (Oishi *et al.*, 1990) and 5-HT_{1A}-receptors (Oishi *et al.*, 1992).

1.4. H_3-Heteroreceptors

H_3-Receptors are widely distributed in the CNS and their localization is not confined to histaminergic neurons. With the use of the H_3-receptor agonist (R)α-methylhistamine and the H_3-antagonist thioperamide it was shown that H_3-heteroreceptors are able to modulate the release of different neurotransmitters i.e. noradrenaline (Schlicker *et al.*, 1989), serotonin (Fink *et al.*, 1990; Rodrigues *et al.*, 1995) and dopamine (Schlicker *et al.*, 1993) in rodent brain. Recently, activation of H_3-receptors was shown to inhibit [^{3}H]noradrenaline release from the human cerebral cortex (Schlicker, 1996).
H_3-Receptors are also present in several peripheral tissues like the airways (Ichinose & Barnes, 1989; Ichinose *et al.*, 1989; Burgaud & Oudart, 1993; Danko *et al.*, 1994; Delaunois *et al.*, 1995), the cardiovascular system (Ea-Kim & Oudart, 1988; Hey *et al.*, 1992; Ea-Kim *et al.*, 1993; Gothert *et al.*, 1995), the stomach (Bertaccini *et al.*, 1991; Coruzzi *et al.*, 1991; Hollande *et al.*, 1993; Bertaccini & Coruzzi, 1995) the intestine (Trzeciakowski, 1987; Hew *et al.*, 1990; Coruzzi *et al.*, 1991; Leurs *et al.*, 1991; Schwörer *et al.*, 1992; Vollinga *et al.*, 1992) and the retina (Schlicker *et al.*, 1990). Also in the periphery, modulation of release of the previously mentioned neurotransmitters (Schlicker *et al.*, 1994c), of acetylcholine (Tamura *et al.*, 1988; Poli *et al.*, 1991) and of neuropeptides (Matsubara *et al.*, 1992; Imamura *et al.*, 1996) seems to be an important mechanism underlying H_3-receptor mediated effects. H_3-Heteroreceptors were demonstrated on nerve terminals of postganglionic parasympathetic and sympathetic nerves and on NANC neurons (C-fibers). Additionally, H_3-receptors appeared also to be located on non-neuronal cells, e.g. intestinal enterochromaffin cells (Schwörer *et al.*, 1992; Schwörer *et al.*, 1994) and enterochromaffin-like (ECL) cells in the stomach (Bertaccini & Coruzzi, 1995).

1.5. Postsynaptic H_3-receptors

More recently, receptor autoradiographic studies in rat brain provided evidence for the existence of postsynaptic H_3-receptors (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). Destruction of postsynaptic structures of the striatum with the neurotoxins quinolinic acid or kainic acid resulted in a marked reduction of striatal H_3-receptor binding. As the majority of the striatal efferents use γ-aminobutyric acid (GABA) as their neurotransmitter H_3-receptors in the striatum may be largely located on GABA neurons, projecting to other parts of the basal ganglia. Postsynaptic H_3-receptors might also exist in other components of the basal ganglia. Lesions of the dopaminergic system increased H_3-receptor binding in the substantia nigra (Ryu *et al.*, 1994; this thesis, Chapter 6). In addition, altered H_3-receptor binding was found in the globus pallidus (decreased binding) and the entopeduncular nucleus (increased binding), following neurochemical destruction of the dopaminergic system (see Chapter 6). The changed H_3-receptor binding in the basal ganglia after destruction of dopaminergic neurons with 6-hydroxydopamine showed a pattern similar to the changed binding of $GABA_A$-receptors, which are predominantly of postsynaptic origin. Hence, H_3-receptors in the basal ganglia might in part be co-localized with $GABA_A$-receptors. Postsynaptic H_3-receptors may also be present in other brain areas. Electrocoagulation of the lateral hypothalamus resulted in an increased [^{3}H](R)α-methylhistamine binding in the rat cerebral cortex (Pollard *et al.*, 1993). Its must be noted though that evidence for the existence of postsynaptic H_3-receptors is mainly based on receptor binding studies. The functional role of postsynaptic H_3-receptor is yet to be explored.

1.6. The H_3-receptor as a regulatory entity in histaminergic signal transduction

The discovery of the histamine H_3-receptor has provided new perspectives in histamine research. As the autoreceptor of histaminergic neurons, the histamine H_3-receptor may play a pivotal role in the modulation of processes related to histaminergic neuronal activity in the brain. Both, H_3-heteroreceptors and postsynaptic H_3-receptors may play an important role in the cross-talk of the histaminergic system with other neurotransmitter systems in the brain. Especially in peripheral tissues, histamine H_3-receptors are also a putative target for histamine released from non-neuronal stores such as mast cells, basophils, enterochromaffin cells and adrenal chromaffin cells (Tuominen *et al.*, 1993; Dimitriadou *et al.*, 1994; Ohkubo *et al.*, 1994; Bertaccini & Coruzzi, 1995). The affinity of histamine for H_3-receptors is about 100-fold higher than its affinity for H_1- and

H_2-receptors. This makes the histamine H_3-receptor a primary target in tissues where all three subtypes of histamine receptors co-exist.

1.7. The second messenger system of the H_3-receptor

Up till now, attempts to identify the H_3-receptor gene have not been successful. Hence, information on the transductional mechanism of H_3-receptors obtained so far is based on pharmacological studies performed in membrane fractions and from functional receptor assays.

The H_3-receptor has been partly purified from bovine brain. Digitonin-solubilization from bovine brain homogenates yielded a molecular mass of 220kDa, probably representing the receptor coupled to a co-solubilized G-protein (Zweig *et al.*, 1992). A more accurate estimation of the H_3-receptor protein mass has been obtained in the human gastric tumor cell line HGT-1 (Cherifi *et al.*, 1992). The H_3-receptor has been purified to a degree of 84% from these cells. SDS-PAGE revealed a molecular mass of 70kDa (Cherifi *et al.*, 1992).

Several lines of evidence have been obtained for the involvement of G-proteins in H_3-receptor mediated signal transduction. H_3-Receptor binding studies performed in rat and bovine brain revealed the sensitivity of agonist binding to guanine nucleotides (West *et al.*, 1990b; Arrang *et al.*, 1990; Zweig *et al.*, 1992; Jansen *et al.*, 1994; Ligneau *et al.*, 1994). Also, H_3-agonists were shown to stimulate the binding of GTPγS to rat cerebral cortical membranes (Clark & Hill, 1996). Moreover, the H_3-receptor mediated stimulation of GTPγS binding was abolished by pertussis toxin (Clark & Hill, 1996). In the mouse pituitary cell line AtT-20, pertussis toxin pretreatment decreased H_3-receptor binding, which was associated with the ADP ribosylation of a 41kDa protein (Clark *et al.*, 1993; West *et al.*, 1994).

The involvement of G-proteins in H_3-receptor mediated signal transduction has also been indicated from functional studies. Pertussis toxin attenuated the H_3-receptor mediated inhibition of the adrenergic responses of the guinea-pig atrium (Endou *et al.*, 1994), [^{3}H]noradrenaline release from rat spinal cord slices (Celuch, 1995), sympathetic neurotransmission in guinea-pig perivascular mesenteric arteries (Ishikawa & Sperelakis, 1987) and cholinergic transmission in the guinea-pig duodenum (Poli *et al.*, 1993). In HGT-1 cells, H_3-receptor mediated inhibition of inositol phosphates formation was sensitive to both cholera toxin and pertussis toxin treatment (Cherifi *et al.*, 1992). A pertussis toxin insensitive GTP binding protein was suggested to be involved in H_3-receptor mediated augmentation of Ca^{2+} currents in the rabbit saphenous artery (Oike *et al.*, 1992). Hence, different G-protein subfamilies might be involved in H_3-receptor

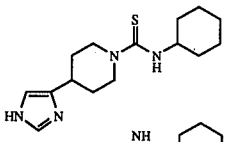
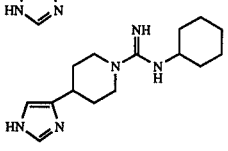
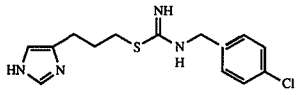
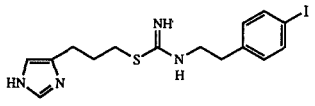
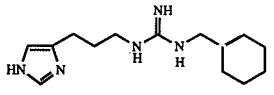
mediated signal transduction. Most of the studies cited indicate the coupling of H_3-receptors to $G_{i/o}$-proteins however.

Yet, the second messenger of the H_3-receptor is not well understood. It seems unlikely that H_3-receptors are directly coupled to adenylate cyclases (Garbarg *et al.*, 1989; Clark *et al.*, 1993; Poli *et al.*, 1993; Schlicker *et al.*, 1994a). In AtT-20 cells, attempts to show an H_3-receptor mediated modulation of phospholipase A_2, C or D and efforts to modulate Ca^{2+} or cyclic nucleotide levels were reported not to be successful (Clark *et al.*, 1993). Cherifi *et al.* provided evidence for a negative coupling of H_3-receptors to phospholipase C in HGT-1 cells (Cherifi *et al.*, 1992). The H_3-agonists (R)α-methylhistamine and Nα-methylhistamine inhibited the basal release of inositol triphosphate from the gastric tumor cells (Cherifi *et al.*, 1992). The inhibition by (R)α-methylhistamine was antagonized by thioperamide. In addition, (R)α-methylhistamine counteracted the carbachol stimulated inositol triphosphate formation. Thus, H_3-receptors in HGT-1 cells might be directly coupled to phospholipase C (Cherifi *et al.*, 1992).

Several reports have described an effect of ion channel modulators on H_3-receptor mediated effects. Inhibition by H_3-agonists of [^{3}H]noradrenaline release from mouse cerebral cortex slices was attenuated by the K^+-channel blocker tetraethylammonium (TEA), but was unaffected by the ATP-sensitive K^+-channel blocker glibenclamide (Schlicker *et al.*, 1994a). The effect of TEA was dependent on the calcium concentration in the superfusion medium. It was suggested that H_3-receptor stimulation modulated ion channels via indirect mechanisms (Schlicker *et al.*, 1994a). Differently from the study described by Schlicker and coworkers, H_3-receptors in the rat hindpaw were proposed to be coupled to ATP-sensitive K^+-channels, based on the attenuation by glibenclamide of the H_3-receptor mediated inhibition of substance P released from sensory nerve endings (Ohkubo & Shibata, 1995). Involvement of N-type Ca^{2+}-channels in H_3-receptor activation has been suggested in the guinea-pig heart (Endou *et al.*, 1994), in the guinea-pig intestine (Poli *et al.*, 1994), in the rabbit saphenous artery (Oike *et al.*, 1992) and in the rat brain (Takemura *et al.*, 1989). At present it is however not clear whether the described effects of cation channel modulators on H_3-receptor responses result from a direct coupling of the receptors to these channels or from secondary mechanisms.

H_3-Receptor activation has also been indicated to stimulate the release of nitric oxide in the rabbit cerebral artery (Kim *et al.*, 1992), in the guinea-pig cardiovascular system (Ea-Kim *et al.*, 1993; Ea-Kim *et al.*, 1996) and in guinea-pig bronchioles (Burgaud & Oudart, 1994). The H_3-receptor response in the tissues mentioned was attenuated by inhibitors of nitric oxide synthase. The effects of H_3-receptor ligands on the nitric oxide pathway are likely to be secondary to activation of the H_3-receptor second messenger system.

Table 2. *Structures and potencies of potent histamine H_3-receptor antagonists.*

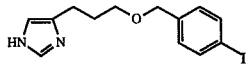
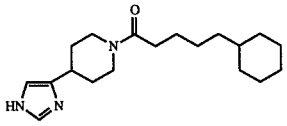
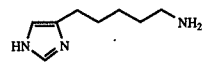
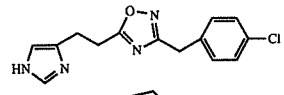
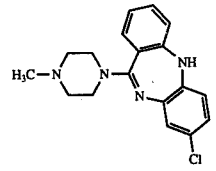
compound	structure	potency	reference
thioperamide		$pA_2 = 8.4$[a] $ED_{50} = 2$ mg/kg[b]	Schwartz, 1990 Garbarg, 1989
GR168320		$pA_2 = 9.7$[c]	Brown, 1996
clobenpropit		$pA_2 = 9.9$[c] $ED_{50} = 11$ mg/kg; rat[d] $ED_{50} = \sim 1$ mg/kg; mouse[e]	Van der Goot, 1992 Barnes, 1993 Yokoyama, 1994
iodophenpropit		$pA_2 = 9.6$[c]	Jansen, 1994
		$pA_2 = 9.2$[f]	Schlicker, 1994 Stark, 1994

[a]Inhibition of neuronal histamine release from rat cerebral cortex; [b]histamine turnover, rat brain, i.p. administration; [c]inhibition of the neurogenic contraction of the guinea-pig intestine; [d]*ex vivo* displacement of [^{3}H]N$^{\alpha}$-methylhistamine from rat brain, s.c. administration; [e]inhibition of electrically induced convulsions in mice, i.p. administration; [f]inhibition of [^{3}H]noradrenaline release from mouse cerebral cortex.

methylhistamine was reported to be substantially improved by the application of azomethine prodrugs of (R)α-methylhistamine (Krause *et al.*, 1995; Krause *et al.*, 1996). These compounds may become valuable tools, both pharmacologically and clinically (Krause *et al.*, 1995).

[^{3}H](R)α-Methylhistamine, [^{3}H]N$^{\alpha}$-methylhistamine and [^{3}H]histamine have been used as radioligands to characterize histamine H_3-receptors in the CNS and in peripheral tissues (see section 3.2).

***Table 2.** (continued).*

compound	structure	potency	reference
iodoproxyfan		$pA_2 = 9.0^g$ $ED_{50} > 10$ mg/kg^h	Ligneau *et al.*, 1994 Stark *et al.*, 1996
GT-2016		$pK_i = 7.4^i$ $ED_{50} \sim 5$ mg/kg^j	Tedford *et al.*, 1995 Tedford *et al.*, 1995
impentamine (VUF4702)		$pA_2 = 8.4^g$	Vollinga *et al.*, 1995
GR175737		$pA_2 = 8.1^g$ $ED_{50} = 1.4$ mg/kg^j	Clitherow *et al.*, 1996 Clitherow *et al.*, 1996
clozapine		$pA_2 = 7.1^k$	Rodrigues *et al.*, 1995

^{g}Inhibition of the neurogenic contraction of the guinea-pig intestine; ^{h}histamine turnover, mouse brain, p.o. administration; ^{i}inhibition of [^{3}H]N$^\alpha$-methylhistamine binding to rat cerebral cortex membranes; ^{j}*ex vivo* displacement of [^{3}H]N$^\alpha$-methylhistamine from rat brain, GT-2016: i.p. administration, GR175737: s.c. administration; ^{k}inhibition of [^{3}H]serotonin release from rat cerebral cortex. Note: impentamine is a partial agonist on the H_3-receptor mediated inhibition of [^{3}H]noradrenaline release from mouse cerebral cortex slices ([Leurs, 1996 #123], pD_2=8.1, i.a.=0.6) Iodoproxyfan has been reported to be a partial agonist on the guinea-pig ileum (Schlicker *et al.*, 1996, neurogenic contraction, pD_2 and intrinsic activity not described) and on the H_3-receptor mediated inhibition of [^{3}H]noradrenaline release from mouse cerebral cortex slices (Schlicker *et al.*, 1996, i.a.=0.3, at 10 nM).

2.2. H_3-Antagonists

Analogous to H_3-agonists, the most potent H_3-antagonists currently known share an imidazole group. The side chain of compounds with H_3-antagonistic activity display an even larger heterogeneity than the side chain of H_3-agonists (see Table 2). In addition to the compounds mentioned in Table 2 other potent antagonists have been found, including carbamates (Stark *et al.*, 1996a; Stark *et al.*, 1996b), esters (Stark *et al.*, 1996a), ethers

(Huls *et al.*, 1996) and amidines (Aslanian *et al.*, 1995).
Most of the mentioned compounds show pA_2-values between 8 and 10 and, as far as described, *in vivo* ED_{50}-values in the range of 1 to 10 mg/kg (Table 2). The classical H_3-receptor antagonist thioperamide is a potent and valuable tool, being widely used for *in vitro* and *in vivo* studies (Arrang *et al.*, 1987a; Leurs & Timmerman, 1992; Onodera *et al.*, 1994). One of the most potent antagonists *in vitro* presently known is clobenpropit (pA_2-value of 9.9), a derivative of the agonist imetit (Van der Goot *et al.*, 1992). Clobenpropit displays a relatively low potency with respect to *in vivo* CNS effects in rats (Barnes *et al.*, 1993; Mochizuki *et al.*, 1996, Chapter 8 of this thesis). In mice however, the compound potently inhibited electrically induced convulsions (Yokoyama *et al.*, 1994). Another highly potent H_3-antagonist *in vitro* is GR168320, in which the thiourea-moiety of thioperamide is replaced by a guanidine group (Brown *et al.*, 1996). Some histamine H_2-receptor ligands possess a moderate antagonistic activity towards histamine H_3-receptors, such as burimamide (pA_2-value of 7.5, Arrang *et al.*, 1983), various burimamide derivatives (Vollinga *et al.*, 1995b) and impromidine (pA_2-value of 7.5, Arrang *et al.*, 1983).
The imidazole moiety is not prerequisite for H_3-antagonistic activity, although the currently known non-imidazole compounds show rather low to moderate potencies. Examples of non-imidazole containing H_3-antagonists are the atypical neuroleptic clozapine (pA_2-value of 7.1, Rodrigues *et al.*, 1995; pA_2-value of 6.3, Kathmann *et al.*, 1994), the H_2-agonist dimaprit (pA_2-value of 5.5, Schwartz *et al.*, 1990) and betahistine (pA_2-value of 5.2, Arrang *et al.*, 1985).
Another interesting compound with a moderate antagonistic activity is verongamine (IC_{50}-value of 0.5 μM) which was isolated from a marine sponge (Mierzwa *et al.*, 1994). Verongamine has been used as a starting compound for the synthesis of the first chiral H_3-antagonists (Tedford *et al.*, 1996). These compounds may become powerful tools in H_3-receptor research, especially with respect to SAR studies.
Although clear H_3-receptor related CNS effects are observed with various H_3-antagonists (see Table 2), only a minor fraction of peripherally injected antagonists penetrates into the brain (Sakurai *et al.*, 1994; Windhorst *et al.*, 1996, Chapter 7 of this thesis). Consequently, also for H_3-antagonists undesired peripheral effects may occur at the doses needed to occupy central H_3-receptors. Hence, the development of H_3-antagonists exhibiting a higher brain penetration is desirable.
The structural diversity of the side chain of compounds with H_3-antagonistic activity provides opportunities for the development of radioligands. In 1992, our research group introduced [^{125}I]iodophenpropit as the first radiolabelled histamine H_3-receptor antagonist (Menge *et al.*, 1992; Jansen *et al.*, 1992). In addition, four other radiolabelled H_3-

antagonists have been described i.e. [^{3}H]S-methylthioperamide (Yanai *et al.*, 1994), [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994), [^{3}H]thioperamide (Alves-Rodrigues *et al.*, 1996) and [^{3}H]GR168320 (Brown *et al.*, 1996). The binding characteristics of these radioligands will be discussed in section 3.3.

3. H_3-Receptor binding studies

3.1. Prelude: selection criteria for radioligands

Receptor radioligands play a crucial role in receptor research, covering many aspects of receptor pharmacology. From *in vitro* receptor binding studies information is obtained on receptor-ligand interactions, tissue distribution of the receptor, G-protein coupling, ion dependence, receptor heterogeneity and isolation and structural identification of the receptor protein. In addition, *in vivo* receptor binding techniques such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) contribute to the unraveling of receptor physiology and pathophysiology.

The main criteria for the selection of a radioligand are summarized in Table 3. Development of a ligand binding assay starts with a ligand (agonist or antagonist) meeting the following basic requirements: a high binding affinity, reversibility, selectivity and an appropriate radiosynthesis route yielding a sufficiently high specific activity (an important determinant in the sensitivity of the assay). Furthermore, the ligand must bind to the tissue preparations in a saturable way. The nonspecific binding, defined as binding of the radioligand to 'non-target-receptor components' should be sufficiently low, yielding a high 'signal-to-noise-ratio'. The two last criteria (saturability and signal-to-noise-ratio) are difficult to predict in advance, as they largely depend on the tissue and on the pharmacodynamical characteristics of the ligand. The fulfilment of these criteria is determined by the relative affinity of the radioligand for the target receptor as compared to the non-target-receptors and by the ratio between the target and the non-target receptor densities in the tissue. Another criterion is that binding of the radioligand must be region or tissue specific and display a heterogeneous subcellular distribution. Finally, binding to the target receptor has to be validated by the correlation of the receptor binding affinities of a preferably heterogeneous group of ligands with their potencies determined in functional studies. Enantiomers exhibiting a clear stereoselectivity for the receptor are also valuable tools in this respect (see also: Laduron, 1988).

Table 3. *Selection criteria for a radioligand for in vitro receptor binding studies.*

basic criteria:
-high affinity
-reversibility
-high selectivity
-high specific activity
additional criteria:
-validation of the 'basic criteria'
-saturability
-proper 'signal to noise ratio', preferably higher than 50%
-heterogeneous tissue distribution
-heterogeneous subcellular distribution
-correlation of binding data with functional data
using a heterogeneous group of ligands
using stereoselective binding tools, if available

From the discovery of the histamine H_3-receptor in 1983 until now, eight different radioligands have been used in H_3-receptor binding studies. The first three ligands described are tritiated agonists. More recently, the availability of different selective H_3-antagonists led to the introduction of three tritiated and of two radioiodinated antagonists. The radiolabelled agonists and antagonists will be successively reviewed in the next sections.

3.2. Radiolabelled H_3-agonists

The tritiated agonists [^{3}H](R)α-methylhistamine, [^{3}H]N$^\alpha$-methylhistamine and [^{3}H]histamine were the first radioligands used to characterize histamine H_3-receptors in different species. In Table 4 the main characteristics of these ligands are summarized. All three ligands were shown to bind reversibly, saturably and with high affinity to H_3-receptors in rat brain membrane preparations. [^{3}H](R)α-Methylhistamine was the first radioligand available (Arrang *et al.*, 1987a). The compound has been used at different laboratories to study H_3-receptor characteristics and distribution from rodents to primates (Arrang *et al.*, 1987a; Martinez-Mir *et al.*, 1990; West *et al.*, 1990a; Fujimoto *et al.*, 1991; Yanai *et al.*, 1992; Pollard *et al.*, 1993).

As mentioned before, N$^\alpha$-methylhistamine has a lower H_3-receptor selectivity as compared to (R)α-methylhistamine (Schwartz *et al.*, 1990; Oishi *et al.*, 1993).

Nevertheless, [^{3}H]N$^\alpha$-methylhistamine has proven to be a very suitable radioligand for H_3-receptor binding studies and it has widely been used (Korte *et al.*, 1990; Cumming *et al.*, 1991; Cherifi *et al.*, 1992; Clark *et al.*, 1993; Kathmann *et al.*, 1993; West *et al.*, 1994). [^{3}H]N$^\alpha$-Methylhistamine has the advantage of a higher specific activity and a lower nonspecific binding as compared to [^{3}H](R)α-methylhistamine (Table 4). [^{3}H]Histamine has a relatively low selectivity towards H_3-receptors and would not be the first choice for H_3-receptor binding studies. A few reports have described its use as a radioligand to study histamine H_3-receptors however (Cumming *et al.*, 1991; Zweig *et al.*, 1992). [^{3}H]Histamine has been successfully used to partially purify the H_3-receptor protein from bovine brain (Zweig *et al.*, 1992). In 1980, Barbin *et al.* found that the binding profile of tritiated histamine to rat brain membranes did not correspond to H_1- and

Table 4. *Characteristics of radiolabelled agonists used to study histamine H_3-receptors.*

	[^{3}H](R)α-MeHA	[^{3}H]N$^\alpha$-MeHA	[^{3}H]histamine
Dissociation constant (K_D, nM)[a]	0.4 - 2.5	0.4 - 2	5 - 8
Receptor density (B_{max}, fmol/mg)[a]	30 - 190	40 - 150	20 - 80
Specific binding (% of total binding)[a]	60 - 90	> 90	~ 75
Specific activity (Ci/mmol)	20 - 40	80	6 - 43
Sensitivity to guanine nucleotides	·	·	·
Functional potency (EC_{50}-value, nM)	4	15	62
Species characterized	rat guinea-pig primate	rat guinea-pig mouse	rat bovine
References used	[1-5]	[6-14]	[8,9]

[1] Arrang *et al.*, 1987a, [2] Arrang *et al.*, 1990, [3] West *et al.*, 1990a, [4] Kilpatrick & Michel, 1991, [5] Yanai *et al.*, 1994, [6] Korte *et al.*, 1990, [7] West *et al.*, 1990b, [8] Cumming *et al.*, 1991, [9] Zweig *et al.*, 1992, [10] Clark *et al.*, 1993, [11] Kathmann *et al.*, 1993, [12] West *et al.*, 1994, [13] Clark & Hill, 1995, [14] Brown *et al.*, 1996. Data are derived from receptor binding studies in the species indicated, using brain membranes preparations (except for Cumming, 1991; autoradiographic study). [a]Values correspond to experiments using rat whole brain or rat cerebral cortex. The specific binding is related to radioligand concentrations around or below the K_D-value. EC_{50}-Values correspond to the inhibition of the potassium induced [^{3}H]histamine release from rat cerebral cortex slices, as taken from Leurs et al. (1992). Abbreviations: [^{3}H](R)α-MeHA, [^{3}H](R)α-methylhistamine; [^{3}H]N$^\alpha$-MeHA, [^{3}H]N$^\alpha$-methylhistamine.

H_2-receptors known at that time (Barbin *et al.*, 1980). Hence, [^{3}H]histamine may be the first radioligand used to 'unintentionally' label H_3-receptors at the time the H_3-receptor had not yet been identified. Nevertheless, [^{3}H]histamine cannot be regarded as a very selective radioligand. At nanomolar concentrations [^{3}H]histamine binds to multiple sites in guinea-pig cerebral cortex membranes (Sinkins *et al.*, 1993; Sinkins & Wells, 1993).
Binding of the three tritiated agonists to brain tissue is sensitive to guanine nucleotides (Table 4) and to pertussis toxin ([^{3}H]N$^{\alpha}$-methylhistamine; Clark *et al.*, 1993), indicating that histamine H_3-receptors, like histamine H_1- and H_2-receptors, belong to the G-protein coupled receptors. Guanine nucleotides were generally shown to inhibit agonist receptor binding (Arrang *et al.*, 1990; West *et al.*, 1990b; Cumming *et al.*, 1991; Kilpatrick & Michel, 1991; Zweig *et al.*, 1992; Clark *et al.*, 1993; Clark & Hill, 1995). The underlying mechanism(s) for the reduction of radiolabelled agonist binding has not been investigated in most studies and is currently not well understood. The reduction of agonist binding may result from both a reduced B_{max} or from a reduced affinity (Clark *et al.*, 1993). It has also been suggested that guanine nucleotides affect a subpopulation of the binding sites (West *et al.*, 1990b). In one report, the guanine nucleotide induced reduction of agonist binding has been described to be dependent on the presence of calcium in the incubation buffer (Arrang *et al.*, 1990). It is obvious that the radiolabelled H_3-agonists do not display a straightforward binding profile with respect to the G-protein coupling and this may partly explain the inconsistencies found in literature.
There is a clear discrepancy between the receptor binding affinities and the functional potencies of H_3-agonists (Table 4). In general, the affinity of agonists observed in binding studies exceeds their functional potency by about 10-fold (Arrang *et al.*, 1990; Schwartz *et al.*, 1990; Leurs *et al.*, 1995b). In contrast, for H_3-antagonists, a good correlation between receptor binding affinity and functional potencies is generally obtained (Kathmann *et al.*, 1993; Schlicker *et al.*, 1994b; Leurs *et al.*, 1995b; see next section). The discrepancy between binding affinities and functional potencies as observed with agonists is likely to result from the involvement of G-protein coupling in agonist binding, i.e. the radiolabelled agonists may bind to the high affinity receptor state predominantly. This phenomenon might also explain the relatively low H_3-receptor densities observed using tritiated agonists (30 to 190 fmol/mg of protein; Table 4) as compared to the densities observed for most radiolabelled antagonists (70 to 400 fmol/mg of protein; Table 5). The involvement of G-protein coupling in agonist binding can be regarded as a general drawback of the use of radiolabelled agonists as tools for receptor binding, complicating the interpretation of the binding data.

3.3. Radiolabelled H_3-antagonists

The complexity of the binding profile of the radiolabelled agonists stressed the need for radiolabelled antagonists for H_3-receptor binding studies. In the last five years two radioiodinated and three tritiated antagonists have been reported. The main characteristics of these ligands are summarized in Table 5. Binding of the antagonists to rat brain show a high affinity, and is saturable and reversible. Like for the agonists, also the radiolabelled antagonists described so far are imidazoles, differing in their side chain (for the chemical structures see section 2.2).

The introduction of [^{125}I]-labelled antagonists has led to probes with a high specific activity, yielding a higher sensitivity as compared to tritiated compounds. The high sensitivity of the radioiodinated probes has been especially beneficial with respect to the exposure time required to obtain receptor binding autoradiograms (i.e. several hours versus several months, using conventional Hyperfilm). It may be noted though that the exposure times of tritiated radioligands could be significantly reduced to several days using the recently developed storage phosphor imaging method, which has been applied to study the autoradiographic distribution of [^{3}H](R)α-methylhistamine and of [^{3}H]S-methylthioperamide (Yanai *et al.*, 1992; Yanai *et al.*, 1994; see also Chapter 5 and Chapter 6 of this thesis).

As shown in Table 5, considerable differences are found between the receptor densities described with the radiolabelled antagonists. Although it may be noted that the values given in Table 5 are not all derived from the same tissue preparation, the tissue variation does not adequately explain the differences in B_{max}-values.

The nonspecific binding of the radiolabelled antagonists appeared to be comparatively high, except for [^{3}H]GR168320. The definition of the nonspecific binding of the radiolabelled antagonists needs critical consideration. For some antagonists, radioligand binding displaced by antagonists was found to exceed radioligand binding displaced by agonists, depending on the brain area and species used. An obvious interpretation of these observations is that the radiolabelled antagonists bind to 'non-H_3-receptor components' from which they are readily displaced by H_3-antagonists, and not by H_3-agonists. The phenomenon has been observed for [^{125}I]iodophenpropit, [^{125}I]iodoproxyfan and for [^{3}H]thioperamide (see Chapter 5; Ligneau *et al.*, 1994; Alves-Rodrigues *et al.*, 1996). For [^{125}I]iodophenpropit, the magnitude of the difference between agonist and antagonist displacement largely varied between different rat brain regions. In most brain areas a difference of 10 to 20% was found between [^{125}I]iodophenpropit binding displaced by the H_3-receptor agonist (R)α-methylhistamine and by the H_3-receptor antagonist thioperamide (Chapter 5). A difference of about 40% between binding displaced between agonists and antagonists has been observed for [^{125}I]iodoproxyfan, in striatal tissue

(Ligneau *et al.*, 1994). Comparable differences were found for [^{3}H]thioperamide binding to rat cerebral cortex membranes (Alves-Rodrigues *et al.*, 1996). From these results it has been concluded that H_3-agonists can generally be regarded as more reliable tools to define the nonspecific binding of the radiolabelled antagonists.
At present, the origin of the non-H_3-receptor binding component(s) of H_3-antagonists is largely unknown. For [^{3}H]thioperamide, binding to cytochrome P_{450} isoenzymes may be involved (Alves-Rodrigues *et al.*, 1996). The same has been suggested for [^{3}H]S-methylthioperamide (Yanai *et al.*, 1994). The origin of the nonspecific binding may of course differ between the radiolabelled antagonists however. Illustrative for this rationale, the thioperamide related compound [^{3}H]GR168320 seems not to exhibit the phenomenon of a differential displacement by agonists and antagonists, at least in rat cerebral cortex membranes (Brown *et al.*, 1996). Recently, iodophenpropit and thioperamide have been screened on a about forty different receptor assays (Leurs *et al.*, 1995a). The screening

Table 5. *Characteristics of radiolabelled antagonists used to study histamine H_3-receptors.*

	[^{125}I]IPP[a]	[^{125}I]IPF[b]	[^{3}H]SMT[c]	[^{3}H]thioperamide[a]	[^{3}H]GR168320[a]
Dissociation constant (K_D, nM)	0.3 - 0.6	0.1	2.1	0.8	0.1
Receptor density (B_{max}, fmol/mg)	250 - 350	78	550	73	412
Specific binding (% of total binding)	45 - 55[d]	50 - 60	70 - 80	50 - 60	>90
Specific activity (Ci/mmol)	2000	2000	50	6	5
Sensitivity to guanine nucleotides		+	N.D.	N.D.	N.D.
Functional potency (K_B-value, nM)	0.3	1 - 5	N.D.		0.2
Species characterized	rat mouse	rat	rat	rat	rat
References used	[1,2]	[3]	[4]	[5]	[6]

[1] Jansen *et al.*, 1992, [2] Jansen *et al.*, 1994, [3] Ligneau *et al.*, 1994, [4] Yanai *et al.*, 1994, [5] Alves-Rodrigues *et al.*, 1996, [6] Brown *et al.*, 1996. Data are derived from receptor binding studies using brain membranes preparations, and values correspond to experiments using [a]rat cerebral cortex, [b]rat striatum or [c]rat forebrain. [d] ~75% as determined in mouse whole brain. The specific binding is related to radioligand concentrations around or below the K_D-value. Functional potencies correspond to the inhibition of the potassium induced [^{3}H]histamine release from rat cerebral cortex slices or to H_3-receptor mediated inhibition of the electrically contracted guinea-pig jejunum. Abbreviations: [^{125}I]IPP, [^{125}I]iodophenpropit; [^{125}I]IPF, [^{125}I]iodoproxyfan; [^{3}H]SMT, [^{3}H]S-methylthioperamide; N.D., not described.

did not yield a possible candidate for the nonspecific binding of both radioligands. Both iodophenpropit and thioperamide display a relatively high affinity for $5HT_3$-receptors (K_i-values of 11 nM and 120 nM, respectively). At the experimental conditions used, $5HT_3$-receptor binding does not interfere with the the assay for both radioligands however. The relatively high nonspecific binding of most radiolabelled antagonists can be regarded as a drawback. In this respect, [^{3}H]GR168320 may be the most appropriate radioligand. However, its low specific activity of 4.8 Ci/mmol seems to be inadequate for the generation of autoradiographic images.

Consistent with conventional models of antagonist receptor binding, saturation binding curves of [^{125}I]iodophenpropit were not affected by guanine nucleotides. Displacement of [^{125}I]iodophenpropit by H_3-agonists was usually biphasic in nature and displacement curves were shifted to the right (towards a monophasic curve) after inclusion of GTPγS in the incubation medium, consistent with an involvement of G-proteins in the binding of the agonists (Jansen *et al.*, 1994; Leurs *et al.*, 1996; Chapter 3 and 4). In contrast to [^{125}I]iodophenpropit, binding of [^{125}I]iodoproxyfan to rat striatal membranes was shown to be partly sensitive to guanine nucleotides (Ligneau *et al.*, 1994). This observation might be related to the partial agonistic nature of iodoproxyfan recently described (Schlicker *et al.*, 1996). Also for [^{125}I]iodoproxyfan biphasic agonist displacement binding curves were reported. Histamine competition binding curves were shifted to the right by guanine nucleotides (Ligneau *et al.*, 1994). The effect of guanine nucleotides on the binding curves of the tritiated antagonists has not been investigated so far. Tritiated antagonists yielded biphasic agonist competition binding curves. Competition binding curves of (R)α-methylhistamine were shown not to be affected by GTPγS, using [^{3}H]thioperamide as a radioligand (Alves-Rodrigues *et al.*, 1996). This rather unexpected finding might result from a changed G-protein coupling at the relatively low temperature (i.e. 4°C) used in the study (Alves-Rodrigues *et al.*, 1996). Altogether, the radiolabelled antagonists provided further evidence for an involvement of G-proteins in H_3-receptor mediated signal transduction. However, not all the antagonists display a 'classical' behaviour regarding the sensitivity towards guanine nucleotides.

Using radiolabelled antagonists, the affinities of different unlabelled antagonists generally correlated well with their functional potencies (pA_2-values). For [^{3}H]S-methylthioperamide a detailed binding analysis including competition binding curves of different ligands has not been presented however. Considering competition binding curves of agonists, with [^{125}I]iodophenpropit, [^{3}H]thioperamide and [^{3}H]GR168320, a good correlation of the high affinity binding site of agonists with the EC_{50}-values found in functional studies has been described (Jansen *et al.*, 1994; Alves-Rodrigues *et al.*,

1996; Brown *et al.*, 1996). For [^{125}I]iodoproxyfan, agonist affinities obtained from competition binding experiments were 3 to 10-fold higher as compared to their functional potencies. In this respect, [^{125}I]iodoproxyfan displays characteristics comparable to radiolabelled agonists (see previous section).

To resume, the introduction of radiolabelled antagonists has provided useful new tools for H_3-receptor binding studies. A good correlation has generally been obtained between binding of unlabelled H_3-antagonists and their functional potencies. For most of the radiolabelled antagonists, the same holds true with respect to agonist competition binding curves, implicating a lack of involvement of G-protein coupling in the binding of the radioligands themselves. However, the guanine sensitivity of binding of tritiated antagonists has largely to be explored. Further studies are also required to elucidate the origin of the nonspecific binding observed for most radiolabelled antagonists.

3.4. Distribution of H_3-receptor binding sites in the CNS

H_3-Receptor radioligands have provided important information on the localization of H_3-receptors in the brain. The autoradiographic distribution of H_3-receptor binding sites has been studied in rat brain using [^{3}H](R)α-methylhistamine (Arrang *et al.*, 1987a; Yanai *et al.*, 1992; Pollard *et al.*, 1993; Ryu *et al.*, 1995), [^{3}H]N$^{\alpha}$-methylhistamine (Cumming *et al.*, 1991; Cumming *et al.*, 1994a) and more recently with [^{125}I]iodophenpropit (Jansen *et al.*, 1994), [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994) and [^{3}H]S-methylthioperamide (Yanai *et al.*, 1994). In contrast to the differences in binding characteristics between the H_3-receptor radioligands found using brain membrane preparations, a consistent overlap is observed with respect to the autoradiographic distribution of the radioligand binding sites.

A comprehensive study on the distribution of H_3-receptors in the rat CNS has been described by Pollard *et al.*, using [^{3}H](R)α-methylhistamine (Pollard *et al.*, 1993). H_3-Receptor binding sites in rat brain are heterogeneously distributed. Highest densities are observed in the cerebral cortex, the olfactory tubercles, the caudate putamen, the nucleus accumbens and the substantia nigra. Moderate densities are found in the hippocampus, the globus pallidus, the thalamus and the hypothalamus, including the histaminergic perikarya in the posterior area. Densities are low in the cerebellum and in the brain stem. In some brain areas a laminar distribution of H_3-receptor binding sites is found. For example, in the cortex highest densities are observed in the deeper layers (IV-VI). The hippocampus displays a relatively dense labelling in the dentate gyrus and the subiculum (Pollard *et al.*, 1993).

Yet no substantial species differences have been found for the H_3-receptor distribution, in contrast to marked species differences previously described for H_1- and H_2-receptors (Garbarg *et al.*, 1992). Beside the rat, the distribution of H_3-receptors has been examined in guinea-pig, mouse and primate brain. In guinea-pig (Cumming *et al.*, 1994b) and mouse forebrain (Cumming *et al.*, 1994b; see also Chapter 4), a distribution is found similar to the distribution described in the rat. The guinea-pig shows relatively low densities in the striatum as compared to the cerebral cortex. In mouse brain, relatively high densities were observed in the globus pallidus, as compared to the rat globus pallidus (see Chapter 4). Receptor autoradiographic studies have also been performed in monkey and in human brain (Martinez-Mir *et al.*, 1990). These two species showed a distribution comparable to rodents. However, in primates, the highest H_3-receptor densities were found in the basal ganglia, especially in the medial and lateral segments of the globus pallidus.

H_3-Receptor binding sites in the CNS display a distribution pattern distinct from the localization of the histaminergic varicosities. Considering the existence of H_3-heteroreceptors, this finding is not unexpected. Functional studies have indicated the presynaptic localization of H_3-receptors on noradrenergic (Schlicker *et al.*, 1989), and on serotonergic (Fink *et al.*, 1990; Rodrigues *et al.*, 1995) nerve terminals in the cerebral cortex. In addition, H_3-receptor activation also inhibited the release of acetylcholine from the entorhinal cortex (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995). The inhibition of acetylcholine release was not found in synaptosomes and may therefore occur either via presynaptic regulation of non-cholinergic neurotransmitter release or via postsynaptic mechanisms (Arrang *et al.*, 1995). Receptor binding studies provided evidence for a presynaptic localization of H_3-receptors on GABA neurons in the substantia nigra (Cumming *et al.*, 1991; Ryu *et al.*, 1994). Chemical destruction of striatal GABA neurons terminating in the substantia nigra, resulted in a decrease of H_3-receptor binding sites in the substantia nigra (Cumming *et al.*, 1991; Ryu *et al.*, 1994). The presynaptic localization of H_3-receptors in the substantia nigra has recently been confirmed in superfused rat brain slices, demonstrating an inhibition by H_3-agonists of dopamine D_1-receptor stimulated GABA release (Garcia *et al.*, 1997).

Receptor binding studies have also indicated a postsynaptic localization of H_3-receptors. Chemical destruction of postsynaptic structures in the striatum using quinolinic and kainic acid resulted in a marked decrease of striatal H_3-receptor binding sites (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). A major part of the striatal H_3-receptors may be located on striatal GABA neurons, representing more than 85% of the striatal efferents (Kita & Kitai, 1988). H_3-Receptors in the striatum may be functionally linked to dopaminergic neurons. Lesion of dopaminergic neurons originating from the substantia

nigra increased H_3-receptor densities in the striatum, especially in the dorsal aspects (Ryu *et al.*, 1994; see also Chapter 6). These observations indicated that striatal H_3-receptors are not predominantly located on dopaminergic neurons, in line with an indicated postsynaptic localization. An interaction between H_3-receptors and the dopaminergic system has also been indicated in other components of the basal ganglia. Lesion of nigrostriatal dopaminergic neurons also increased H_3-receptor densities in the substantia nigra (Ryu *et al.*, 1994; Ryu *et al.*, 1996). The increased binding in the substantia nigra was reversed by striatal application of the D_1-receptor agonist SKF38393 indicating a functional role of dopamine and of striatonigral GABA neurons in H_3-receptor regulation in the substantia nigra (Ryu *et al.*, 1996). Additionally, lesions of the rat dopaminergic system changed H_3-receptor binding in the globus pallidus and in the entopeduncular nucleus (see Chapter 6). The H_3-receptors in the different basal ganglial areas mentioned may be in part of postsynaptic origin.

Increased H_3-receptor binding in the striatum and in the cerebral cortex after lesions of the medial forebrain bundle at the level of the hypothalamus has also indicated a possible link between H_3-receptors and other aminergic transmitters, including histamine (Pollard *et al.*, 1993). Accordingly, selective depletion of neuronal histamine with the histidine decarboxylase inhibitor α-fluoromethylhistamine increased H_3-receptor binding in the striatum and in the substantia nigra (Ryu *et al.*, 1995; Ryu *et al.*, 1996). Treatment of rats with reserpine resulted in a marked reduction of H_3-receptor binding in the nucleus accumbens and the striatum (Cumming & Vincent, 1993). In contrast, H_3-receptor binding in the surrounding cortical areas remained unchanged. The authors suggested that the decreased H_3-receptor binding in the striatal areas may result from an increased histamine synthesis and consequent increase of histamine release, induced by reserpine treatment (Cumming & Vincent, 1993). Visual deprivation by enucleation of the rat eye, resulted in a marked increase (50 - 100%) of H_3-receptor binding in the superior colliculus, the area receiving the major part of the retinal efferents (Nakagawa *et al.*, 1994). Increased H_3-receptor binding was also observed in the visual cortex following visual deprivation (Nakagawa *et al.*, 1994). Resuming, several autoradiographic studies have reported an increase of H_3-receptor binding following a loss of neuronal input (Ryu *et al.*, 1996).

3.5. H_3-Receptor binding studies in peripheral tissues

The densities of H_3-receptors in the periphery appeared to be much lower as compared to densities in the CNS. This makes peripheral H_3-receptors less accessible for receptor binding studies, and consequently explains that only a limited number of studies on H_3-

receptor binding in peripheral tissues has been described so far. H_3-Receptors in the periphery of the guinea-pig have been characterized with [^{3}H]N$^\alpha$-methylhistamine (Korte *et al.*, 1990). In most tissues H_3-receptor densities were below 1 fmol/mg of protein. Highest densities (between 4 and 8 fmol/mg protein) were found in the large intestine, the ileum, the pancreas and the pituitary. A full pharmacological characterization of the [^{3}H]N$^\alpha$-methylhistamine binding sites in the peripheral tissues was not presented (Korte *et al.*, 1990).

H_3-Receptors were also detected in the human gastric mucosa (Courillonmallet *et al.*, 1995). [^{3}H]N$^\alpha$-Methylhistamine saturation binding to mucosal H_3-receptors yielded a receptor density of 10 fmol/mg of protein. H_3-Receptor binding was reduced in Heliobacter *pylori* infected patients (Courillonmallet *et al.*, 1995). Gastric H_3-receptors have also been characterized using a human fundic tumor cell line (HGT-1). Binding of [^{3}H]N$^\alpha$-methylhistamine to these cells was sensitive to GTPγS and to both cholera and pertussis toxin, again indicating the coupling of the gastric H_3-receptors to G-proteins (Cherifi *et al.*, 1992). Similar results have been obtained in the murine pituitary tumor cell line AtT-20 (Clark *et al.*, 1993; West *et al.*, 1994). [^{3}H]N$^\alpha$-Methylhistamine binding was sensitive to GTPγS and to pertussis toxin, indicating the coupling to G-proteins of the $G_{i/o}$ subfamily (Clark *et al.*, 1993). In guinea-pig lung the distribution of H_3-receptors has been visualized by receptor autoradiography (Schwartz *et al.*, 1990). [^{3}H](R)α-Methylhistamine binding was scattered in the parenchyma. A more dense labelling was observed in the bronchioles (Schwartz *et al.*, 1990).

Except for [^{3}H]S-methylthioperamide, receptor binding studies to peripheral tissues have not been described for radiolabelled antagonists. [^{3}H]S-Methylthioperamide showed a considerably high amount of nonspecific binding, which interfered with the accurate determination of H_3-receptors in peripheral tissues (Yanai *et al.*, 1994). Based on the relatively high amount of nonspecific binding observed with most radiolabelled H_3-antagonists, similar limitations may evolve for other radiolabelled antagonists.

3.6. Heterogeneity of binding sites

3.6.1. Radiolabelled H_3-agonist binding sites

In 1990, West *et al.* reported that thioperamide and burimamide discriminated [^{3}H]N$^\alpha$-methylhistamine binding to rat brain membranes into high and low affinity binding sites (West *et al.*, 1990b). [^{3}H]N$^\alpha$-Methylhistamine binding was partly decreased by the GTP analogue GTPγS. In the presence of GTPγS, thioperamide and burimamide yielded monophasic competition binding curves, with affinities corresponding to their high

affinity binding sites. From these results the existence of subtypes of H_3-receptors i.e. H_{3A}- and H_{3B}-receptors was proposed, the latter being sensitive towards guanine nucleotides (West *et al.*, 1990b). [^{3}H]N$^{\alpha}$-Methylhistamine itself did not discriminate between the proposed H_{3A}- and H_{3B}-receptors.

In a study by Arrang and co-workers, using the agonist [^{3}H](R)α-methylhistamine as the radioligand, biphasic competition binding curves in rat cerebral cortex membranes were obtained for burimamide, but not for thioperamide (Arrang *et al.*, 1990). A guanine nucleotide sensitivity of the burimamide binding sites was not reported in this study. In contrast to [^{3}H]N$^{\alpha}$-methylhistamine (West *et al.*, 1990b), in the standard incubation medium, binding of [^{3}H](R)α-methylhistamine was not sensitive to the GTP analogue Gpp(NH)p. However, when calcium was added to the incubation buffer, two binding sites were found for [^{3}H](R)α-methylhistamine, the low affinity site being abolished by Gpp(NH)p (Arrang *et al.*, 1990). From these observations it may be suggested that the possible heterogeneity of burimamide binding sites and of [^{3}H](R)α-methylhistamine binding sites are unrelated phenomena. The heterogeneity of [^{3}H](R)α-methylhistamine binding sites was suggested to result from the conversion of a subpopulation of the receptors into low-affinity binding sites, triggered by calcium (Arrang *et al.*, 1990).

A heterogeneity of [^{3}H](R)α-methylhistamine binding sites has also been found in kinetic studies, using buffer without calcium (West *et al.*, 1990a). In this study a homogeneous population of [^{3}H](R)α-methylhistamine binding sites was observed at equilibrium conditions (i.e. saturation binding analysis) however. Thioperamide and burimamide yielded monophasic competition binding curves in this report (West *et al.*, 1990a).

The three reports cited illustrate the complexity of the receptor binding data obtained with the radiolabelled agonists and the controversies in literature with respect to a heterogeneity of H_3-receptor binding sites. Biphasic competition binding curves for burimamide have been described in several reports using [^{3}H](R)α-methylhistamine (Arrang *et al.*, 1990) and [^{3}H]N$^{\alpha}$-methylhistamine (West *et al.*, 1990b; Kathmann *et al.*, 1993; Cumming & Gjedde, 1994; Brown *et al.*, 1996). Accordingly, different studies reported a heterogeneous displacement of [^{3}H]N$^{\alpha}$-methylhistamine by thioperamide (West *et al.*, 1990b; Cumming & Gjedde, 1994; Clark & Hill, 1995; Brown *et al.*, 1996). Controversially, other studies did not confirm the presence of two distinct binding sites for burimamide (West *et al.*, 1990a; Kilpatrick & Michel, 1991; Clark & Hill, 1995) and for thioperamide (Arrang *et al.*, 1990; West *et al.*, 1990a; Kilpatrick & Michel, 1991; Kathmann *et al.*, 1993).

One explanation for the different observations concerning heterogeneity of thioperamide and burimamide binding sites is the relatively small difference in affinity between the two separate binding sites, making it difficult to discriminate them statistically. In addition, the controversies concerning the heterogeneity of binding sites may arise from different

experimental conditions used, like the choice of buffer (Tris-HCl, phosphate, HEPES), the ionic composition of the buffer (mono- and divalent cations) and the tissue preparation used (cerebral cortex versus whole brain). For example, it has been reported that the affinity of thioperamide for [^{3}H](R)α-methylhistamine binding sites was 10-fold higher in phosphate buffer as compared to Tris-HCl buffer (West *et al.*, 1990a). In contrast the affinity of the agonists histamine, (R)α-methylhistamine and N$^\alpha$-methylhistamine were not substantially different when phosphate and Tris-HCl buffer are compared (Arrang *et al.*, 1987a; West *et al.*, 1990a).

Also, the ionic composition of the buffer has been indicated to differentially affect binding characteristics of ligands. As previously cited, guanine nucleotide sensitivity of [^{3}H](R)α-methylhistamine (but not of [^{3}H]N$^\alpha$-methylhistamine; West *et al.*, 1990b) was dependent on the presence of calcium in the buffer (Arrang *et al.*, 1990). Sodium ions were shown to abolish the low affinity binding site of thioperamide, whereas the binding affinities of clobenpropit and N$^\alpha$-methylhistamine were not affected (Clark & Hill, 1995). From these results, it was suggested that the H_3-receptor exists in different conformations, for each of which thioperamide has a different affinity (Clark & Hill, 1995).

Hence, contribution of differential allosteric effects dependent of the buffer composition may relate to the observed heterogeneity of binding sites and to the controversy in literature in this respect. A differential allosteric action of sodium has also been reported for other receptor systems including the binding of H_1-receptor antagonists (Treherne *et al.*, 1991; Gibson *et al.*, 1994). The allosteric effect may also be related to the involvement of G-proteins in the binding of agonists to the receptor, further complicating the interpretation of the binding data. Altogether, the complexity of the binding profile of radiolabelled agonists does not provide a sound basis for the definition of H_3-receptor subtypes. The necessity for the validation of the possible heterogeneity of H_3-receptors by functional pharmacological studies clearly emerges.

3.6.2. Radiolabelled H_3-antagonist binding sites

From a theoretical viewpoint, radiolabelled antagonists are expected to display a more straightforward binding profile lacking effects of receptor - G-protein interaction on binding of the radioligands themselves. In section 3.3 it has already been indicated that not all antagonists currently known fit into the conventional receptor model however.

For all radiolabelled antagonists, biphasic competition binding curves were reported for H_3-agonists (Jansen *et al.*, 1994; Yanai *et al.*, 1994; Ligneau *et al.*, 1994; Alves-Rodrigues *et al.*, 1996; Brown *et al.*, 1996). In the [^{125}I]iodophenpropit and [^{125}I]-iodoproxyfan binding assays, agonist competition binding curves were sensitive to

guanine nucleotides (Jansen *et al.*, 1994; Ligneau *et al.*, 1994). The apparent heterogeneity of agonist binding may therefore be attributed to the involvement of G-proteins in the agonist receptor binding rather than to a receptor heterogeneity. (Jansen *et al.*, 1994; Chapter 3). For the tritiated antagonists, the sensitivity to guanine nucleotides was not studied or could not be demonstrated at the experimental conditions used (Alves-Rodrigues *et al.*, 1996).

[^{125}I]Iodophenpropit was biphasically displaced from rat cortex membranes by the antagonists burimamide and dimaprit (Jansen *et al.*, 1994; Chapter 3). In contrast to agonist binding, antagonist binding was not affected by GTPγS. Hence, biphasic competition binding curves of burimamide and dimaprit were likely not related to the G-protein coupling of the [^{125}I]iodophenpropit binding sites. So far, a heterogeneous distribution of putative H_3-receptor subtypes has not been demonstrated. Using a receptor autoradiographic approach, [^{125}I]iodophenpropit binding to ten different rat brain areas was not discriminated by a chemically heterogeneous group of H_3-receptor antagonists (see Chapter 5). For the other radiolabelled antagonists, biphasic competition binding curves of antagonists have so far not been demonstrated. Remarkably, thioperamide and burimamide yielded steep competition binding curves (Hill-coefficients of 1.7 and 1.9, respectively) in rat striatal membranes using the [^{125}I]-iodoproxyfan assay (Ligneau *et al.*, 1994).

Yet, it can be concluded that receptor binding studies with radiolabelled antagonists did not reveal clear evidence for H_3-receptor heterogeneity. In general, the exploration of H_3-receptor subtypes requires the availability of ligands with a higher selectivity towards one of these putative subtypes.

3.6.3. Evidence for receptor heterogeneity from functional studies

A few reports described the putative existence of H_3-receptor heterogeneity based on functional studies. H_3-Receptors inhibiting acetylcholine release from rat entorhinal cortex slices were proposed to be pharmacologically distinct from the 'classical H_3-receptors' mediating the inhibition of neuronal histamine release (Clapham & Kilpatrick, 1992). Phenylbutanoylhistamine and betahistine, exhibiting a rather week antagonistic potency for the H_3-receptors mediating the inhibition by (R)α-methylhistamine of neuronal histamine release (Arrang *et al.*, 1985; Clapham & Kilpatrick, 1992), failed to antagonize the inhibition of acetylcholine release from the entorhinal cortex (Clapham & Kilpatrick, 1992). The lack of antagonism by phenylbutanoylhistamine and betahistine was also reported in the periphery, i.e. on the H_3-receptor mediated inhibition of the NANC contractions of the guinea-pig intestine (Taylor & Kilpatrick, 1992) and the inhibition of serotonin release from the porcine enterochromaffin cells (Schwörer *et al.*,

1994). In addition, also impromidine and burimamide failed to antagonize the H_3-receptor mediated inhibition of serotonin release from the pig intestine (Schwörer *et al.*, 1994).
The involvement of an H_3-receptor subtype in the deviating potency of phenylbutanoylhistamine has recently been questioned. Arrang *et al.* showed that micromolar concentrations of phenylbutanoylhistamine inhibited acetylcholine release, by a non-histaminergic mechanism (Arrang *et al.*, 1995). It was therefore suggested that the deviating potency of phenylbutanoylhistamine on the acetylcholine release from the entorhinal cortex may be attributed to the limited selectivity of the compound, rather than the existence of H_3-receptor subtypes (Arrang *et al.*, 1995). The same could account for betahistine which is not a histamine H_3-receptor selective compound. Additional results on the deviating potencies of impromidine and burimamide have not been presented so far.
A series of histamine homologues was recently shown to display a discriminative pharmacological behaviour between the functional assay in the jejunum and in the CNS (Leurs *et al.*, 1996). The propylene, butylene and pentylene (impentamine) homologues of histamine were full antagonists on the guinea-pig ileum, but displayed partial agonism in the mouse brain cortex (Leurs *et al.*, 1996). Similar phenomena were recently described for iodoproxyfan and its deiodo analogue (Schlicker *et al.*, 1996). Moreover, a homologue of the H_3-agonist immepip was found to discriminate between H_3-receptors in the guinea-pig jejunum and the rat cerebral cortex (Alves-Rodrigues, 1996). At present, a straightforward interpretation of these data in terms of receptor subtypes, species differences or signal transductional mechanisms is not possible. In this respect, additional potent compounds are desired with an exclusive antagonistic behaviour in different functional models.
The functional studies mentioned above obviously do not provide evidence for the putative existence of H_{3A}- and H_{3B}-receptors as suggested from the biphasic displacement by thioperamide and burimamide of radiolabelled agonists from rat brain (West *et al.*, 1990b). Based on the functional potencies of thioperamide and of tiotidine, H_{3A}- and H_{3B}-receptors were suggested to be linked to H_3-receptor mediated inhibition of histamine release and synthesis, respectively (West *et al.*, 1990b). So far, the existence of putative receptor subtypes has not clearly been further documented. Hence, the existence of H_{3A}- and H_{3B}-receptor subtypes is yet not certain.

3.7. Resumption and concluding remarks

Studies performed with H_3-receptor radioligands have substantially contributed to the current knowledge of the characteristics, distribution and function of the histamine H_3-receptor. Tritiated agonists were successfully used to study H_3-receptors in rodent and

primate CNS. Binding studies with radiolabelled agonists provided evidence for a role of G-proteins in H_3-receptor mediated signal transduction. The apparent involvement of G-protein coupling in the binding of the radiolabelled agonists may underlay two less favourable features of the radioligands however. At first, an overestimation of H_3-agonists potencies is obtained in competition binding studies. Secondly, the complexity of the radiolabelled agonists binding dynamics makes it difficult to distinguish binding phenomena related to G-protein coupling, allosteric interactions, and receptor heterogeneity in terms of H_3-receptor subtypes. Radiolabelled agonists are advantageous with respect to their low nonspecific binding in the rat CNS. [^{3}H](R)α-Methylhistamine and [^{3}H]N$^{\alpha}$-methylhistamine were both shown to be very useful studying the distribution of H_3-receptors by autoradiography.

The introduction of radiolabelled H_3-receptor antagonists yielded improved tools for H_3-receptor binding studies. With the use of these ligands additional evidence was provided for the interaction of H_3-receptors with G-proteins. As compared to radiolabelled agonists, [^{125}I]iodophenpropit, [^{3}H]GR168320 and [^{3}H]thioperamide exhibit the advantage of a good correlation between agonist binding affinities and their functional potencies.

So far, studies with radiolabelled H_3-antagonists did not provide much progress in the search for H_3-receptor heterogeneity. Ligands which more clearly discriminate between putative subtypes are still awaited, and a link between binding heterogeneity and functional receptor responses will be indispensable. Not all radiolabelled antagonists display a straightforward binding profile, which may in part be due to the relatively high amount of nonspecific binding of most antagonists, to be considered as a drawback. In this respect [^{3}H]GR168320 is a promising ligand, displaying a negligible amount of nonspecific binding, allowing a unambiguous interpretation of receptor binding data.

4. Aim and outline of this thesis

When this PhD-project was initiated in 1992, only tritiated agonists i.e. [^{3}H](R)α-methylhistamine, [^{3}H]N$^{\alpha}$-methylhistamine and [^{3}H]histamine were available for histamine H_3-receptor binding studies. In the same year our group introduced [^{125}I]iodophenpropit, the first radiolabelled H_3-receptor antagonist. Iodophenpropit is a member of a series of highly potent isothiourea containing H_3-antagonists, developed at our laboratory. The major part of this thesis deals with the validation of [^{125}I]iodophenpropit as a radioligand for *in vitro* receptor binding experiments studying characteristics, distribution and function of H_3-receptors in the brain.

Chapter 2 describes a preliminary characterization of [^{125}I]iodophenpropit binding to rat cerebral cortex membranes. A comprehensive analysis of [^{125}I]iodophenpropit binding to rat brain is presented in Chapter 3. [^{125}I]Iodophenpropit meets the criteria for a suitable radioligand to study the histamine H_3-receptor in rat cortex membranes. Receptor binding studies with [^{125}I]iodophenpropit provided additional evidence for the interaction of H_3-receptors with G-proteins and also for a heterogeneity of H_3-receptor binding sites. In Chapter 3, the autoradiographic distribution of [^{125}I]iodophenpropit binding sites in rat brain is briefly described.

The mouse has been routinely used for *in vivo* H_3-receptor models. Using these models, a species difference between rat and mouse has emerged for clobenpropit, a close analogue of iodophenpropit. Almost no data were available on H_3-receptor ligand binding dynamics in mouse brain. In Chapter 4 we therefore describe the characterization of H_3-receptors in mouse brain using [^{125}I]iodophenpropit, in view of a possible species difference between H_3-receptors in rat and mouse.

In Chapter 5 the distribution of [^{125}I]iodophenpropit binding sites in rat brain has been visualized in more detail by receptor autoradiography. Receptor binding densities were quantified using storage phosphor autoradiography, a new method in which digitized images are obtained by exposure of radiolabelled brain sections to storage phosphor plates, instead of exposure to conventional films. In the search for H_3-receptor subtypes, a possible heterogeneity of [^{125}I]iodophenpropit binding sites was studied in different brain regions, performing competition binding experiments with a chemically heterogeneous group of H_3-antagonists.

In chapter 6 we investigated the functional link between central H_3-receptors and the nigrostriatal dopaminergic pathway. 6-Hydroxydopamine denervation of rat dopaminergic neurons altered [^{125}I]iodophenpropit binding in different components of the basal ganglia indicating an involvement of H_3-receptors in basal ganglial neurotransmission.

In order to investigate the potential of iodophenpropit and of its derivatives for *in vivo* imaging studies (e.g. PET and SPECT), radioiodinated iodophenpropit was injected into rats (Chapter 7). The distribution of radiolabelled iodophenpropit and of its radioactive metabolites in different rat tissues is quantified and visualized using whole-body autoradiography.

In Chapter 8 the dynamics of *in vivo* neuronal histamine release from the anterior hypothalamic area is studied, using microdialysis. The effect of the novel H_3-agonist immepip and of the antagonist clobenpropit, chemically related to iodophenpropit, on histamine release has been investigated both after local and systemical administration of the drugs.

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- Chapter 2 -

The first radiolabelled histamine H_3-receptor antagonist [^{125}I]iodophenpropit: saturable and reversible binding to rat cortex membranes - *preliminary characterization*

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Abstract

We describe the binding of [^{125}I]iodophenpropit, the first radiolabelled histamine H_3-antagonist, to rat cortex membranes. The binding of [^{125}I]iodophenpropit was selective, saturable, readily reversible, and of high affinity (K_D-value of 0.32 nM; B_{max}-value of 209 fmol/mg of protein). Specific binding at a concentration of 0.3 nM accounted for 45 to 55% of total binding. [^{125}I]Iodophenpropit is a promising ligand for H_3-receptor binding studies.

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Introduction

Both release and synthesis of neuronal histamine in the central nervous system are modulated by a histaminergic autoreceptor characterized as the H_3-receptor (Arrang *et al.*, 1983). Moreover, the H_3-receptor acts as a heteroreceptor on both central and peripheral nerve tissue (Schwartz *et al.*, 1990; Timmerman, 1990).

A number of H_3-selective ligands have meanwhile been developed, including the agonist (R)α-methylhistamine and the antagonist thioperamide (for review see: Schwartz *et al.*, 1990; Timmerman, 1990; Leurs *et al.*, 1995). For H_3-receptor binding studies the tritium-labelled agonists [^{3}H](R)α-methylhistamine (Arrang *et al.*, 1987), [^{3}H]N^{α}-methylhistamine (Korte *et al.*, 1990) and [^{3}H]histamine (Zweig *et al.*, 1992) have been initially used. Although these radioligands proved to be valuable tools to study histamine H_3-receptors, their binding profiles are rather complex, which may partly arise from the involvement of the interaction of the agonist-receptor complex with G-proteins (review see: Leurs *et al.*, 1995). Hence, radiolabelled H_3-antagonists may constitute improved pharmacological tools for H_3-receptor binding studies.

In our laboratory, a new class of H_3-antagonists exhibiting a high affinity and selectivity towards the H_3-receptor has previously been developed (Van der Goot *et al.*, 1992). Among these H_3-antagonists are halogenated compounds which are potential tools for H_3-receptor binding studies. An iodinated member of this series is iodophenpropit which exhibits a high antagonistic activity (pA_2-value of 9.6) and a high selectivity towards the histamine H_3-receptor, its affinity for H_1- and H_2-receptors being more than 1000-fold lower. In this chapter we describe the binding characteristics of [^{125}I]-labelled iodophenpropit, the first radiolabelled H_3-receptor antagonist (Menge *et al.*, 1992) (Figure 1).

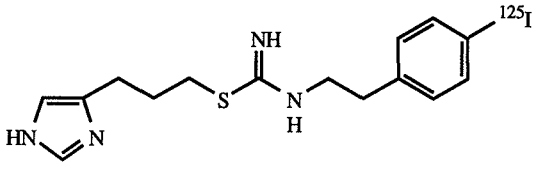

Figure 1. *The chemical structure of [^{125}I]iodophenpropit*

Note: In addition to [^{125}I]iodophenpropit, other radiolabelled histamine H_3-antagonists have more recently been described i.e. [^{125}I]iodoproxyfan, [^{3}H]S-methylthioperamide, [^{3}H]thioperamide and [^{3}H]GR168320. These radioligands are discussed in Chapter 1, 4, 5 and 7.

Materials and Methods

Fresh cerebral cortex membranes from male Wistar rats (200-250 g, Harlan C.P.B., Zeist, The Netherlands) were prepared for each experiment in 50 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 145 mM NaCl and 5 mM $MgCl_2$, by a method essentially according to Leurs *et al.* (1986). [^{125}I]Iodophenpropit (S-[3-(4(5)-imidazolyl)propyl]-N-[2-(4-iodophenyl)ethyl] isothiouronium hydrogen sulfate; specific activity = 1900 Ci/mmol) was synthesized as described by Menge *et al.* (1992).
Binding experiments were performed at 37°C in Tris-HCl buffer (pH 7.4) with a total incubation volume of 0.5 ml, using polyethylene tubes. In saturation and displacement studies, membranes were incubated for 60 minutes. The incubation was started by the addition of 100 µl membranes (20-60 µg/tube) and was terminated by rapid filtration through polyethyleneimine (0.3% w/v) pretreated Whatman GF/C filters, using a Brandel filtration apparatus. The filters were washed twice with 3 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°C). Subsequently, the radioactivity bound to the filters was measured by an LKB gamma counter. (For a more detailed description of the method see Chapter 3)

Results and discussion

At 37°C [^{125}I]iodophenpropit (0.5 nM) rapidly bound to rat cerebral cortex membranes reaching half maximal binding within 1 minute and equilibrium at approximately 30 minutes, which remained stable for at least one additional hour (Figure 2). Specific binding, defined as the difference between total binding and binding in the presence of 0.1 µM thioperamide, was readily reversed by the addition of 1 µM thioperamide (Figure 2). The association and dissociation constants calculated from the kinetic data were 0.75 $nM^{-1}\cdot min^{-1}$ and 0.35 min^{-1} respectively, yielding a K_D-value of 0.47 nM.
Incubation of rat cortex membranes with increasing concentrations [^{125}I]iodophenpropit (0.025 - 5 nM) showed that the specific binding of [^{125}I]iodophenpropit was saturable (Figure 3) and yielded linear Scatchard plots (Figure 4). Computer analysis (Munson & Rodbard, 1980) of the saturation binding curves revealed binding of [^{125}I]iodophenpropit to a single class of sites with a K_D of 0.32 ± 0.06 nM and a B_{max} of 209 ± 53 fmol/mg of protein (n_H = 0.97 ± 0.02, n = 5). Specific binding at a concentration of 0.3 nM accounted for 45 to 55% of total binding. The dissociation constant of [^{125}I]iodophenpropit calculated from the saturation experiments is in agreement with its pA_2-value of 9.6 ± 0.2 as determined on the H_3-receptor mediated relaxation of the electrically contracted guinea-pig jejunum. Displacement of [^{125}I]iodophenpropit (0.25 nM) by the antagonists thioperamide and iodophenpropit was

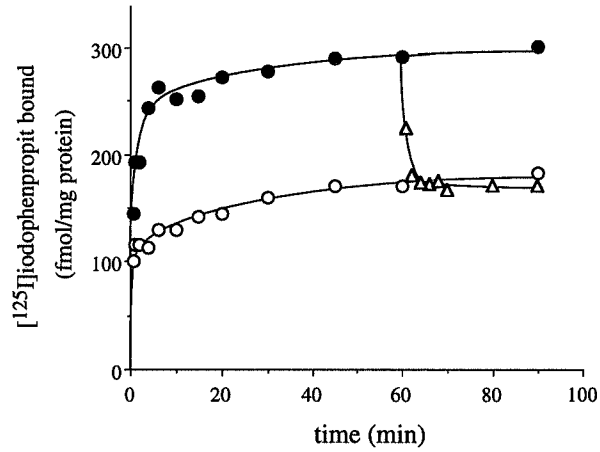

Figure 2. *Kinetics of [^{125}I]iodophenpropit binding to rat cerebral cortex membranes. The association of [^{125}I]iodophenpropit (0.5 nM) was started by addition of the membranes and was measured in the absence (●) and in the presence (○) of thioperamide (0.1 μM), to discriminate between specific and nonspecific binding. Dissociation of [^{125}I]iodophenpropit (Δ) was initiated by the addition of thioperamide (1 μM), after 60 minutes. Data displayed are obtained from a single experiment (of three separate experiments) with triplicate determinations.*

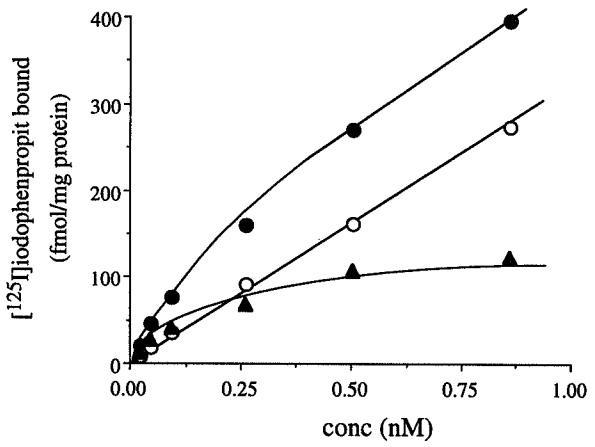

Figure 3. *Saturation binding of [^{125}I]iodophenpropit binding to rat cerebral cortex membranes. Symbols: (●), total binding; (○), nonspecific binding; (▲), specific binding. Nonspecific binding was defined as [^{125}I]iodophenpropit binding in the presence of thioperamide (0.1 μM). Data are obtained from a representative experiment. Each assay was performed with duplicate determinations.*

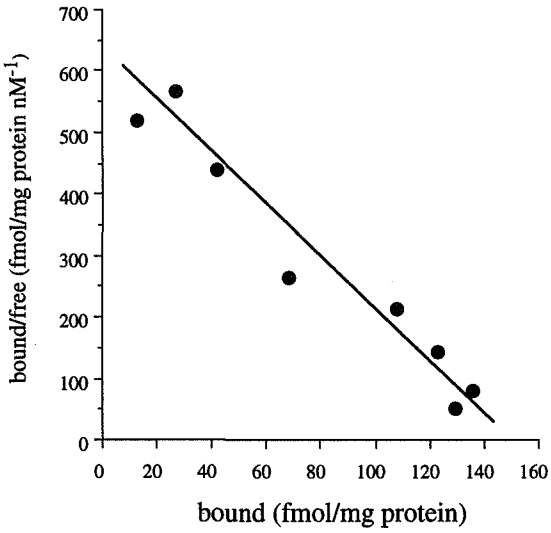

Figure 4. *Scatchard transformation of the [^{125}I]iodophenpropit saturation binding curve.*

best fit by a one site model with K_D- and B_{max}-values of 5.1 ± 1.9 nM and 326 ± 104 fmol/mg of protein for thioperamide (n = 9) and 1.2 ± 0.4 nM and 357 ± 51 fmol/mg of protein for iodophenpropit (n = 6), respectively. In contrast, displacement curves for the agonists histamine and (R)α-methylhistamine were shallow and revealed two binding sites. For histamine the K_H and K_L were 24 ± 16 nM and 1.6 ± 1.2 μM, with a density of 126 ± 59 and 150 ± 46 fmol/mg of protein, respectively (n = 8). (R)α-Methylhistamine showed a K_H and K_L of 4.3 ± 3.4 nM and 0.22 ± 0.15 μM, with corresponding densities of 157 ± 57 and 157 ± 54 fmol/mg of protein, respectively (n = 5). The biphasic displacement of [^{125}I]iodophenpropit by the two agonists may arise from the involvement of G-proteins in agonist binding (see next chapter).

In conclusion, the H_3-antagonist [^{125}I]iodophenpropit seems to fulfill the basic criteria for a suitable ligand for H_3-receptor binding studies, exhibiting a high affinity, saturability, reversibility, selectivity and a high specific activity.

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- *Chapter 3* -

Characterization of the binding of the histamine H_3-receptor antagonist [^{125}I]iodophenpropit to rat brain

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Abstract

We characterized the binding of the selective radiolabelled histamine H_3-receptor antagonist [^{125}I]iodophenpropit to rat cerebral cortex membranes. [^{125}I]Iodophenpropit, radiolabelled to a high specific activity of 1900 Ci·mmol^{-1}, saturably bound to a single class of sites with a K_D of 0.57 ± 0.16 nM (n=4) and a B_{max} of 268 ± 119 fmol·mg^{-1} protein. Specific binding at a concentration below 1 nM represented 50 to 60 % of total binding. Binding of [^{125}I]iodophenpropit to rat cerebral cortex membranes was readily displaced by histamine H_3-agonists and antagonists. In contrast, the inhibitory potencies of selective histamine H_1- and H_2-receptor ligands were very low.

[^{125}I]Iodophenpropit was biphasically displaced by the histamine H_3-receptor antagonists burimamide and dimaprit, which may indicate the existence of histamine H_3-receptor subtypes. Other histamine H_3-receptor antagonists showed a monophasic displacement.

Competition binding curves of H_3-agonists were biphasic and showed a rightward shift upon the addition of the nonhydrolysable GTP analog guanosine 5'-O-(3-thio)triphosphate (GTPγS; 100 μM), implicating the interaction of histamine H_3-receptors with G-proteins. The affinities of the H_3-receptor antagonists iodophenpropit, thioperamide and burimamide were not altered by GTPγS. Histamine competition binding curves were shifted to the right by different nucleotides (100 μM) with a rank order of potency GTPγS > Gpp(NH)p, GTP.

In vitro autoradiographic studies revealed a heterogeneous distribution of [^{125}I]iodophenpropit binding sites in rat brain, with high densities observed in the cerebral cortex, the striatum, the olfactory tubercles, and the substantia nigra.

It is concluded that the histamine H_3-receptor antagonist [^{125}I]iodophenpropit meets the criteria for a suitable radioligand to study histamine H_3-receptors in rat brain.

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Introduction

The histamine H_3-receptor has been characterized as an autoreceptor inhibiting neuronal histamine release in the CNS, and as a heteroreceptor inhibiting the release of various neurotransmitters both in CNS and PNS (Leurs & Timmerman, 1992; Schlicker *et al.*, 1994). Initially, in receptor binding experiments the agonists [^{3}H]histamine, [^{3}H](R)α-methylhistamine and [^{3}H]N$^\alpha$-methylhistamine have been used to study the molecular pharmacology and distribution of histamine H_3-receptors (Arrang *et al.*, 1990; Korte *et al.*, 1990; West *et al.*, 1990a; West *et al.*, 1990b; Kilpatrick & Michel, 1991; Zweig *et al.*, 1992; Clark *et al.*, 1993). These studies suggested the interaction of histamine H_3-receptors with G-proteins (West *et al.*, 1990b; Arrang *et al.*, 1990; Zweig *et al.*, 1992; Clark *et al.*, 1993). Moreover, from studies with [^{3}H]N$^\alpha$-methylhistamine the presence of subtypes has been suggested (West *et al.*, 1990b). However, the binding profile of the radiolabelled agonists appeared to be rather complex. Therefore, the development of radiolabelled histamine H_3-receptor antagonists is of great importance.
In 1992, a new class of highly potent histamine H_3-receptor antagonists has been described (Van der Goot *et al.*, 1992). Its most potent member is clobenpropit (VUF9153) showing a pA_2-value of 9.9 as determined on the electrically contracted guinea-pig intestine. The series allowed the development of the first radiolabelled histamine H_3-receptor antagonist [^{125}I]iodophenpropit (Menge *et al.*, 1992). Preliminary results indicated that [^{125}I]iodophenpropit is a promising tool for histamine H_3-receptor binding experiments (Jansen *et al.*, 1992; Chapter 2). In this chapter we describe the full characterization of [^{125}I]iodophenpropit as a radioligand to study histamine H_3-receptors.

Methods

Preparation of rat cerebral cortex membranes
Male Wistar rats (200-250 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were rapidly removed. The cerebral cortices were dissected and homogenized in 15 volumes (v/w) of ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) using an Ultra-Turrax homogenizer (8 seconds) and a glass-teflon homogenizer (four up and down strokes) subsequently. All

Note: In addition to [^{125}I]iodophenpropit, other radiolabelled histamine H_3-antagonists have more recently been described i.e. [^{125}I]iodoproxyfan, [^{3}H]S-methylthioperamide, [^{3}H]thioperamide, and [^{3}H]GR168320. These radioligands are discussed in Chapter 1, 4, 5 and 7.

subsequent steps preceding incubation were performed at a temperature of 0 to 4°C. The homogenate was centrifuged at 800 g for ten minutes. The pellet was discarded and the supernatant was centrifuged for 20 minutes at 40,000 g. The resulting pellet was resuspended and the last centrifugation step was repeated. The pellet was resuspended in 1.5 volumes (v/w) Tris-HCl buffer and stored at -80°C. Before each receptor binding experiment the membranes were resuspended in Tris-HCl buffer and were centrifuged for 20 minutes at 40,000 g. Finally, the pellet was resuspended in 7 volumes (v/w) of incubation buffer (50 mM Tris-HCl containing 5 mM $MgCl_2$, 145 mM NaCl ; pH 7.4 at 37°C. In some experiments NaCl was omitted).

Receptor binding experiments

Binding experiments were performed at 37°C in the Tris-HCl incubation buffer (pH 7.4) with a total incubation volume of 0.5 ml, using polyethylene tubes. Determinations were performed in triplicate. Drugs (except for thioperamide, see "drugs and chemicals") were prepared in incubation buffer. Rat cerebral cortex membranes were incubated for 60 minutes to reach equilibrium (Jansen *et al.*, 1992; Chapter 2). In saturation experiments membranes were incubated with [^{125}I]iodophenpropit in final concentrations ranging from 0.025 to 3 nM. In competition binding experiments a concentration of 0.25 nM [^{125}I]iodophenpropit was used. Specific binding was defined as the difference between total binding and binding in the presence of 0.3 µM thioperamide and represented more than 50 to 60 % of total binding, at [^{125}I]iodophenpropit concentrations below 1 nM. [^{125}I]Iodophenpropit was displaced to about the same level by all of the histamine H_3-receptor ligands tested. Moreover, at this level [^{125}I]iodophenpropit binding was not further displaced upon the addition of 0.1 µM thioperamide.

Incubations were started by the addition of 100 µl membranes (20 - 60 µg of protein per tube) and were terminated after 60 minutes by adding 2 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°C) immediately followed by filtration through Whatman GF/C filters using a Brandel filtration apparatus. Filters were pretreated (for two hours) with 0.3% polyethyleneimine, reducing filter binding to less than 1 % of the total radioactivity added. After filtration of the membranes, the filters were washed twice with 2 ml of ice-cold Tris-HCl buffer. The amount of radioactivity bound to the membranes was not reduced by repetition of the washing procedure up to five times. The radioactivity bound to the filters was measured by an LKB gamma counter.

Data analysis

Saturation and competition binding experiments were evaluated using the non-linear curve fitting program LIGAND (Munson & Rodbard, 1980) on a Macintosh computer. With

the aid of this program binding curves were fit (unweighed) to a one and a two site model respectively, and statistically tested on the increasing goodness of the fit for a model with additional parameters, based on the 'extra sum of squares' principle (Draper & Smith, 1966), using a probability level of 5%.

Protein assays

Protein concentrations were determined using the Bio-Rad Protein Assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard.

Receptor autoradiography

Male Wistar rats (200-250 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were removed. Brains were frozen using liquid carbon dioxide. Cryostate sections (14 μm) were mounted on gelatine/chromalum-coated glass slides and stored at -80°C until use. The brain sections were thawed and subsequently incubated with 0.3 nM [^{125}I]iodophenpropit for 60 minutes at 37°C in 50 mM Tris-HCl buffer containing 145 mM NaCl, 5 mM $MgCl_2$ and 0.25% BSA (pH 7.4 at 37°C). Non-specific binding was determined by incubation of adjacent sections in the presence of 0.3 μM thioperamide. To stop the incubation, the slices were washed twice for 15 minutes in ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) and 15 seconds in ice-cold distilled water. Sections were dried by a stream of cold air and were exposed to Hyperfilm, (Amersham International, U.K.) for 20 hours.

Drugs and chemicals

[^{125}I]Iodophenpropit was labelled to a specific activity of 1900 Ci mmol^{-1} as described by Menge *et al.* (1992). The radiolabelled compound was stored at 4°C in a 1 mM H_2SO_4 solution in ethanol. H_2SO_4 did not affect the pH of the Tris-HCl buffer at the final concentrations of the radioligand used. HPLC-Analysis of the stock solution of [^{125}I]iodophenpropit two months after preparation revealed that the radioactivity was almost quantitatively present in one peak with a retention time corresponding to the radioligand.

The following drugs and chemicals were used: thioperamide (Schering Corporation), iodophenpropit dihydrobromide (laboratory stock), clobenpropit dihydrobromide (VUF9153, laboratory stock), impromidine trihydrochloride (SK&F Laboratories, UK), burimamide (SK&F Laboratories), dimaprit dihydrochloride (SK&F Laboratories), histamine dihydrochloride (Sigma, St. Louis, USA), (R)α-methylhistamine maleate (gift from Prof.Dr. J-C. Schwartz, Paris), (S)α-methylhistamine dihydrobromide (Cookson Chemicals, St. Louis, USA), imetit dihydrobromide (VUF8325, laboratory stock), immepip dihydrobromide (VUF4708, laboratory stock), 5'-guanylylimidodiphosphate

(Gpp(NH)p, Sigma), guanosine 5'-O-(3-thio)triphosphate (GTPγS, Sigma), guanosine 5'-triphosphate (GTP, Sigma), betahistine dihydrochloride (Solvay Duphar, Weesp, Netherlands), mepyramine hydrochloride (Sigma), triprolidine hydrochloride (Sigma), amthamine dihydrobromide (laboratory stock), tiotidine (ICI, Macclesfield, U.K.), imidazole (Merck) and polyethylenimine (Aldrich, Zwijndrecht, Netherlands). Drugs were dissolved in distilled water or in Tris-HCl buffer except for thioperamide which was diluted in buffer from 1 mM stock solutions prepared in DMSO. At the final concentrations used, DMSO did not affect [125I]iodophenpropit receptor binding.

Statistical analysis

Dissociation constants for agonists determined in the absence and presence of guanine nucleotides were compared using a one tailed unpaired Student's t-test. The effect of NaCl on dissociation constants of antagonists was analyzed by a two tailed unpaired Student's t-test. Differences were considered using a probability level of 5%.

Results

[125I]Iodophenpropit saturation binding experiments

Specific binding of [125I]iodophenpropit (0.025 - 3 nM) to rat cerebral cortex membranes was saturable (Figure 1A) and yielded linear Scatchard plots (not shown). Computer

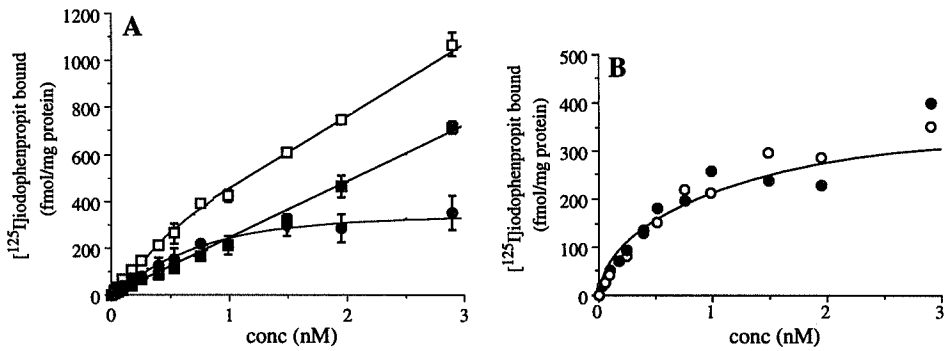

Figure 1. *(A) Saturation binding of [125I]iodophenpropit (0.025 - 3 nM) to rat cerebral cortex membranes. (□) Total binding; (■) non-specific binding; (●) specific binding, determined by the addition of 0.3 μM thioperamide. (B) Specific binding of [125I]iodophenpropit to rat cerebral cortex membranes in the absence (○) and presence (●) of 100 μM Gpp(NH)p. The dissociation constant and receptor density in the absence of Gpp(NH)p were 0.57 ± 0.16 nM and 268 ± 119 fmol·mg^{-1} protein, and in the presence of presence Gpp(NH)p 0.55 ± 0.19 nM and 272 ± 101 fmol·mg^{-1} protein, respectively (n=4). Results displayed are from one representative experiment with triplicate determinations.*

analysis of the saturation binding curves revealed that [^{125}I]iodophenpropit bound to a single class of sites with a K_D of 0.57 ± 0.16 nM and a B_{max} of 268 ± 119 fmol·mg^{-1} of protein (n = 4). Hill coefficients were not significantly different from unity (1.00 ± 0.02). Non-specific binding determined in the presence of 0.3 μM thioperamide was linear with the [^{125}I]iodophenpropit concentration up to 3 nM. Specific binding at concentrations below 1 nM accounted for more than 50 to 60% of total binding.
Both, dissociation constant and receptor density of the [^{125}I]iodophenpropit binding sites were not affected by the non-hydrolysible GTP analog 5'-guanylylimidodiphosphate (Gpp(NH)p) at a concentration of 100 μM (K_D = 0.55 ± 0.19 nM; B_{max} = 272 ± 101 fmol·mg^{-1} protein; n = 4) (Figure 1B).

Competition binding curves of H_3-antagonists

[^{125}I]Iodophenpropit was displaced from rat cerebral cortex membranes by histamine H_3-receptor antagonists (Figure 2). The selective histamine H_3-receptor antagonists clobenpropit (VUF9153) and iodophenpropit showed the highest potency with K_D-values of 0.9 ± 0.4 nM and 1.0 ± 0.1 nM respectively (Table 1). Omitting sodium chloride from the buffer significantly reduced the affinity of iodophenpropit and thioperamide for [^{125}I]iodophenpropit binding sites (Table 1). Competition binding curves of clobenpropit,

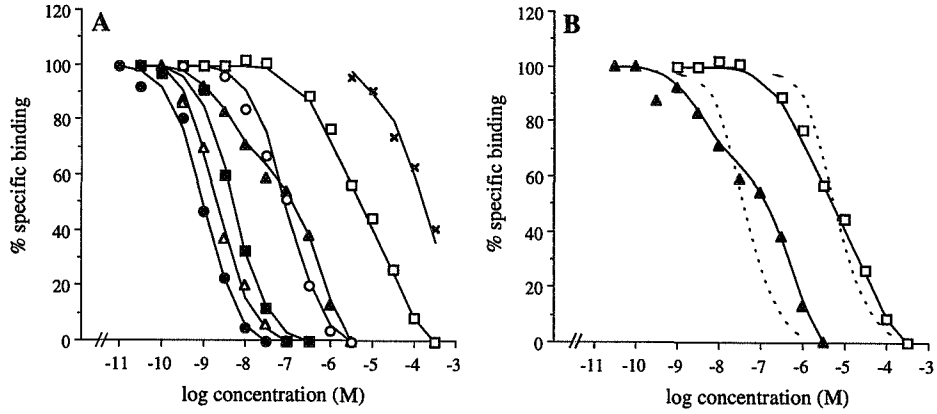

***Figure 2.** (A) Inhibition of [^{125}I]iodophenpropit binding (0.25 nM) by histamine H_3-receptor antagonists. (B) Competition binding curves for burimamide and dimaprit compared with the corresponding theoretical one site competition binding curves (dashed curves). Data are expressed as % of the specific binding. Specific binding was determined using 0.3 μM thioperamide and represented 50 to 60% of the total binding. Each curve represents a single representative experiment with triplicate determinations. Symbols used: (●) clobenpropit, (Δ) iodophenpropit, (■) thioperamide, (○) impromidine, (▲) burimamide, (□) dimaprit, (×) betahistine.*

iodophenpropit, thioperamide (K_D = 4.3 ± 1.6 nM) and impromidine (K_D = 51 ± 9 nM) were best fit according to a one site model (P > 0.05). In contrast, competition binding curves of the histamine H_3-receptor antagonists burimamide and dimaprit were significantly better described by a two site model (P < 0.05; Figure 2B). The dissociation constants for the two different sites were 18 ± 9 nM and 725 ± 392 nM for burimamide and 0.42 ± 0.11 μM and 38 ± 37 μM for dimaprit (Table 2). The histamine H_3-receptor antagonist betahistine had a K_D-value of 131 ± 37 μM (Figure 2A).

Table 1. *Affinities of different ligands towards [125I]iodophenpropit binding sites on rat cerebral cortex membranes and the effect of NaCl on histamine H_3-receptor antagonist affinity.*

ligand	Tris-buffer + $MgCl_2$ (5 mM)	Tris-buffer + $MgCl_2$ (5 mM) + NaCl (145 mM)
H_3-antagonists:	K_D (nM)	K_D (nM)
clobenpropit	N.D.	0.9 ± 0.4 (3)
iodophenpropit	1.7 ± 0.3 (3)	1.0 ± 0.1* (3)
thioperamide	11 ± 2.3 (7)	4.3 ± 1.6* (7)
impromidine	107 ± 68 (3)	51 ± 9 (3)
other ligands:		
mepyramine	> 10,000 (5)	N.D.
triprolidine	> 10,000 (3)	N.D.
tiotidine	> 10,000 (4)	N.D.
amthamine	> 10,000 (2)	N.D.
imidazole	> 10,000 (2)	N.D.

All compounds displayed were best fit according to a one-site model (P > 0.05). Values are given as the mean ± s.d. (number of experiments). N.D.; not determined. * P < 0.05 as compared with buffer without NaCl.

Displacement by other ligands

Selective ligands for histamine H_1- (mepyramine and triprolidine) and H_2-receptors (tiotidine and amthamine) and imidazole showed a very low affinity (K_D > 10 μM) towards the [125I]iodophenpropit binding sites (Table 1).

Table 2. Affinities of histamine H_3-receptor antagonists discriminating between [^{125}I]iodophenpropit binding sites.

antagonist:				
burimamide	$K_{D,1}$ (nM)	R_1 (%)	$K_{D,2}$ (nM)	R_2 (%)
	18 ± 9	54 ± 10	725 ± 392	46 ± 9
dimaprit	$K_{D,1}$ (μM)	R_1 (%)	$K_{D,2}$(μM)	R_2(%)
	0.42 ± 0.11	52 ± 6	38 ± 37	47 ± 6

Values are given as the mean ± s.d. of eight (burimamide) and five (dimaprit) separate experiments with triplicate determinations. $K_{D,1}$ and $K_{D,2}$ are the dissociation constants for the two different binding sites, R_1 and R_2 the corresponding percentages of each site.

[^{125}I]Iodophenpropit displacement by H_3-agonists; the effect of guanine nucleotides

Competition binding curves for histamine H_3-receptor agonists were shallow and were all best fit according to a two site model ($P < 0.05$), showing high and low affinity binding sites. The rank order of potency of the five agonists used for the high affinity sites was imetit (K_H = 2.7 ± 0.8 nM), immepip (K_H = 2.7 ± 0.5 nM), (R)α-methylhistamine (K_H = 3.5 ± 1.2 nM) > histamine (K_H = 38 ± 10 nM) > (S)α-methylhistamine (K_H = 230 ± 97 nM) (Table 3).

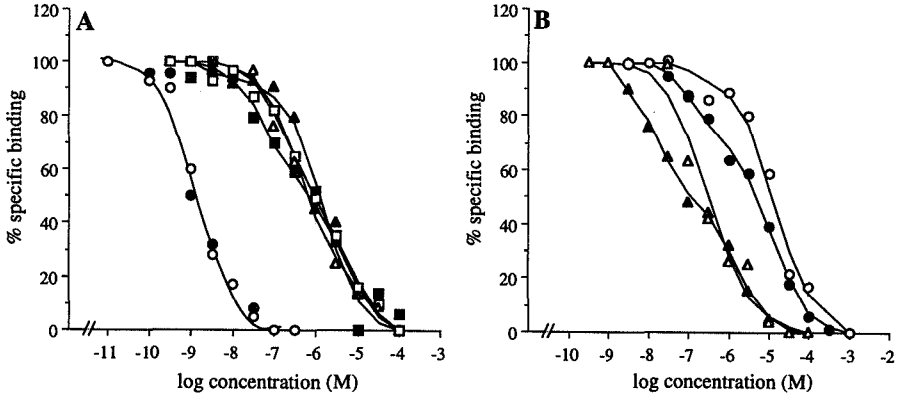

***Figure 3.** The effect of different nucleotides on displacement of [^{125}I]iodophenpropit (0.25 nM). (A) The effect of nucleotides on histamine (HA) and thioperamide competition binding curves. (●) thioperamide, (○) thioperamide + GTPγS, (■) HA, (△) HA + GTP, (□) HA + Gpp(NH)p, (▲) HA + GTPγS. (B) The effect of GTPγS on (R) and (S)α-methylhistamine competition binding curves; (▲) (R)α-methylhistamine, (△) (R)α-methylhistamine + GTPγS, (●) (S)α-methylhistamine, (○) (S)α-methylhistamine + GTPγS. Curves displayed are from representative experiments, each point determined in triplicate. The concentration of the nucleotides used was 100 μM.*

***Table 3.** The effect of guanine nucleotides on the high and low affinity binding sites of histamine H_3-receptor agonists.*

		high affinity		*low affinity*	
agonist	nucleotide	K_H (nM)	R_H (%)	K_L (μM)	R_L (%)
histamine	-	38 ± 10	52 ± 4	2.5 ± 0.6	48 ± 4
	GTP	85 ± 35*	51 ± 8	2.5 ± 0.9	49 ± 8
	Gpp(NH)p	137 ± 100*	42 ± 13	3.2 ± 1.5	58 ± 13
	GTPγs	-	-	0.8 ± 0.1*	100**
(R)α-methylhistamine	-	3.5 ± 1.2	52 ± 4	1.2 ± 0.3	48 ± 3
	GTPγs	-	-	0.1 ± 0.1*	100**
(S)α-methylhistamine	-	230 ± 97	39 ± 11	9.5 ± 1.8	61 ± 5
	GTPγs	-	-	12 ± 3.9	100**
imetit	-	2.7 ± 0.8	59 ± 5	40 ± 12	41 ± 2
	GTPγs	18 ± 10*	52 ± 14	35 ± 20	48 ± 13
immepip	-	2.7 ± 0.5	63 ± 3	1.0 ± 0.2	37 ± 2
	GTPγs	20 ± 5*	67 ± 5	3.4 ± 3.4	33 ± 8

Values are given as the mean ± s.d. of six (histamine ± Gpp(NH)p) or four (remaining) separate experiments with triplicate determinations. The concentrations of GTP, Gpp(NH)p and GTPγs were 100 μM. K_H and K_L correspond to the dissociation constants for the high and low affinity binding sites respectively. R_H and R_L are the percentages of high and low affinity sites. * $P < 0.05$ as compared to value without the nucleotide. ** Curve best fit according to one-site model ($P>0.05$).

Upon the addition of GTPγS (100 μM) the competition binding curves of the agonists showed a rightward shift. GTPγS completely abolished the high affinity sites for histamine, (R)α-methylhistamine and (S)α-methylhistamine (curves were best fit to a one site model, $P > 0.05$; Table 3, Figure 3). For imetit and immepip a significant increase of the high affinity dissociation constant was observed upon addition of GTPγS (Table 3). With respect to competition binding curves for histamine the nucleotides GTP and Gpp(NH)p at a concentration of 100 μM were less potent than GTPγS. Both GTP and Gpp(NH)p significantly increased the high affinity dissociation constant for histamine (Table 3).

GTPγS (100 μM) did not affect the dissociation constants for the histamine H_3-receptor antagonists iodophenpropit (K_D-value without GTPγS: 1.4 ± 0.4 nM, K_D-value with

GTPγS: 2.2 ± 0.8 nM, n = 3), thioperamide (K_D-value without GTPγS: 1.2 ± 0.4 nM, K_D-value with GTPγS: 1.3 ± 0.7 nM, n = 3) and burimamide ($K_{D,1}$-and $K_{D,2}$- value without GTPγS: 18 ± 9 nM and 0.7 ± 0.4 μM respectively, n = 8; $K_{D,1}$ and $K_{D,2}$ -value with GTPγS: 62 ± 44 nM and 3.1 ± 2.8 μM, respectively, n = 3).

Distribution of [^{125}I]iodophenpropit binding sites in rat brain studied by receptor autoradiography

Incubation of rat brain cryostate sections with [^{125}I]iodophenpropit showed that the radioligand binding sites were heterogeneously distributed (Figure 4A). Non-specific binding, defined by incubation of adjacent sections in the presence of 0.3 μM thioperamide, was low and homogeneously distributed (Figure 4B). The highest levels of specific binding were observed in the upper frontal layers and lower temporal layers of the cerebral cortex, the caudate-putamen complex, the nucleus accumbens, the olfactory tubercles and the substantia nigra. A moderate density was observed in the hypothalamic area, the hippocampal formation, the amygdala complex, and the mammillary bodies. Densities were low in the cerebellum.

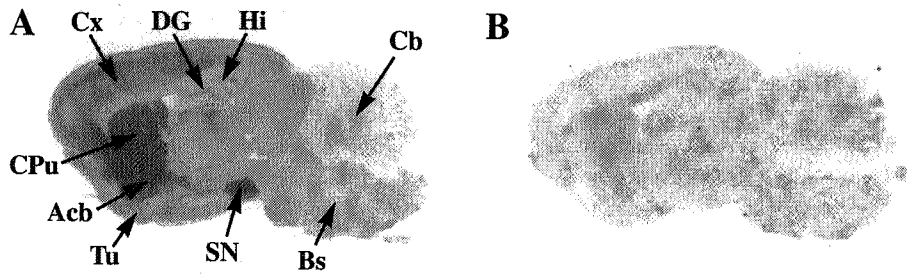

Figure 4. *Autoradiographic localization of [^{125}I]iodophenpropit binding sites in rat brain. Shown is a representative sagittal section (14 μm).* **(A)** *total binding of [^{125}I]iodophenpropit;* **(B)** *non-specific binding, as defined by the inclusion of 0.3 μM thioperamide. Indicated areas: Acb; nucleus accumbens, Bs; brain stem, Cb; cerebellum, Cpu; caudate-putamen, Cx; cortex , DG; dentate gyrus, Tu; olfactory tubercle, Hi; hippocampal formation, SN; substantia nigra.*

Discussion

A preliminary pharmacological characterization of the radiolabelled histamine H_3-receptor antagonists [^{125}I]iodophenpropit indicated that the radioligand is a potential tool for histamine H_3-receptor binding studies (Jansen *et al.*, 1992; Chapter 2). In the present Chapter, we further characterized the binding of [^{125}I]iodophenpropit to rat brain.

[^{125}I]Iodophenpropit meets the basic criteria for a suitable radioligand for receptor binding studies, i.e. high affinity, saturability, reversibility, selectivity and high specific activity. The pK_D-value (9.3) observed in saturation binding experiments is close to the pA_2-value (9.6) determined on the guinea-pig intestine. Binding of the radioligand to histamine H_3-receptors was validated by competition binding studies using various selective histaminergic ligands and by receptor autoradiography.

The binding results obtained with [^{125}I]iodophenpropit largely correlate to data obtained from receptor binding studies using the radiolabelled agonists [^{3}H]histamine, [^{3}H]N$^{\alpha}$-methylhistamine and [^{3}H](R)α-methylhistamine, however also some clear discrepancies exist. The receptor density found using [^{125}I]iodophenpropit (268 fmol·mg^{-1} protein) was 2 to 8-fold higher than the densities reported using radiolabelled agonists (30 to 130 fmol·mg^{-1} protein). The observation that the histamine H_3-receptor antagonists clobenpropit, iodophenpropit, thioperamide and impromidine displaced [^{125}I]iodophenpropit according to a one site model is a clear indication that the displaced binding represents histamine H_3-receptors. Considering the variation in chemical structure of the four ligands mentioned it seems unlikely that the relatively high densities observed using [^{125}I]iodophenpropit are explained by binding of the radioligand to an additional high affinity binding component in the membrane preparation for which all these compounds have the same affinity as for the histamine H_3-receptor. Another indication that the specific [^{125}I]iodophenpropit binding sites represent histamine H_3-receptors is the stereoselective displacement by (R)α-methylhistamine and (S)α-methylhistamine. The differences between B_{max}-values obtained with the currently used radiolabelled H_3-agonists compared with the B_{max}-values obtained with [^{125}I]iodophenpropit might be the result of differences in receptor isolation and incubation procedures. Also, it may be possible that the radiolabelled H_3-agonists bind predominantly to the high affinity state (i.e. G-protein coupled fraction) of histamine H_3-receptors.

Displacement of [^{125}I]iodophenpropit by the histamine H_3-receptor antagonists burimamide and dimaprit was shown to be biphasic. This phenomenon may be due to discrimination between subtypes of histamine H_3-receptors by these two compounds. A two site displacement of radiolabelled histamine H_3-agonists by burimamide in rat brain has previously been reported using [^{3}H](R)α-methylhistamine (rat cerebral cortex membranes; Arrang *et al.*, 1990) and [^{3}H]N$^{\alpha}$-methylhistamine (whole rat brain membranes, West *et al.*, 1990b; rat cerebral cortex, Kathmann *et al.*, 1993). However, in another report using [^{3}H](R)α-methylhistamine (whole rat brain membranes, West *et al.*, 1990a) burimamide displacement was monophasic. A two site displacement has also been reported for thioperamide (West *et al.*, 1990b; Clark & Hill, 1995), but was not observed in other studies. Variation in the experimental conditions (membrane

preparation, composition of the buffer) might account for the discrepancies. Considering the different results with respect to the biphasic displacement of radioligands by H_3-antagonists, the existence of histamine H_3-receptor subtypes from receptor binding studies needs to further be explored. To validate the existence of possible subtypes, more ligands are needed which clearly discriminate between possible subtypes.

The present study provides additional evidence for the interaction of histamine H_3-receptors with G-proteins. Displacement of [^{125}I]iodophenpropit by histamine H_3-receptor agonists was biphasic and was modified by guanine nucleotides. Hence, the two sites found for agonists is probably related to the formation of a ternary complex between the agonist, the receptor and a G-protein rather than discrimination between receptor subtypes by these agonists. The observation that biphasic competition binding curves for the histamine H_3-receptor antagonist burimamide was not significantly affected by GTPγS is in accordance with this conclusion. The results obtained with [^{125}I]iodophenpropit to characterize histamine H_3-receptors were comparable to those from binding studies to other G-protein coupled receptors, such as α- (Weinshank *et al.*, 1990) and β-adrenoceptors (Voss *et al.*, 1992), histamine H_1- (Hattori *et al.*, 1991) and histamine H_2-receptors (Ruat *et al.*, 1990), using radiolabelled antagonists. Generally, guanine nucleotides bind to G-proteins, inducing the uncoupling of the G-protein-receptor complex. In a receptor binding study this shows up as a conversion of agonist high affinity binding sites into low affinity binding sites, since the high affinity binding sites represent the binding of an agonist to the receptor - G-protein complex and the low affinity binding sites represent the binding of an agonist to the receptor alone. In our experiments GTPγS completely abolished the high affinity sites for histamine, (R)α-methylhistamine and (S)α-methylhistamine. However, for imetit and immepip, a significant increase of the high affinity dissociation constant was observed upon addition of GTPγS. Similar results have been described for α_2-adrenoceptors (Weinshank *et al.*, 1990). These observations are different from the generally observed reduction of the ratio between the amount of high and low affinity binding sites by guanine nucleotides. At present, the explanation for these differences remains to be established.

The interaction of histamine H_3-receptors with G-proteins has also been indicated by binding studies using the tritium labeled agonists histamine, (R)α-methylhistamine and N^{α}-methylhistamine. In 1980, Barbin *et al.* described the high affinity binding of [^{3}H]histamine to rat brain. Although at that time histamine H_3-receptors were not yet identified, the [^{3}H]histamine binding sites presumably represented histamine H_3-receptors. The density of the [^{3}H]histamine high affinity binding sites was reduced by Gpp(NH)p without altering the affinity (Barbin *et al.*, 1980). In a study of West and colleagues, a reduction of [^{3}H]N^{α}-methylhistamine binding by GTPγS was observed

which may be due both to a reduced density and affinity of [^{3}H]N$^\alpha$-methylhistamine binding sites (West *et al.*, 1990b). In this last study biphasic displacement of the radiolabelled agonist by thioperamide and burimamide was observed. As the low affinity sites of both antagonists were abolished by GTPγS it was concluded that [^{3}H]N$^\alpha$-methylhistamine bound to two subtypes of histamine H_3-receptors (H_{3A} and H_{3B}) of which one was not detectable in the presence of GTPγS. This conclusion is not in agreement with the results of our experiments using [^{125}I]iodophenpropit, as GTPγS had no effect on displacement curves of burimamide. In another report an effect of guanine nucleotides on [^{3}H](R)α-methylhistamine binding was observed only when calcium was present in the incubation medium (Arrang *et al.*, 1990). When calcium was included in the buffer, saturation binding curves showed a high and low affinity component, the latter being abolished by Gpp(NH)p. From these rather unexpected results the authors suggested that the low affinity site may be its functional receptor. Meanwhile, the guanine nucleotide sensitivity of radiolabelled agonist binding has been described in several reports (West *et al.*, 1990b; Arrang *et al.*, 1990; Kilpatrick & Michel, 1991; Zweig *et al.*, 1992; Clark *et al.*, 1993; Clark & Hill, 1995). In general, these studies showed an inhibitory effect of guanine nucleotides on radiolabelled agonist receptor binding. Reduction of [^{3}H]N$^\alpha$-methylhistamine binding sites by Gpp(NH)p has also been shown by *in vitro* receptor autoradiography (Cumming *et al.*, 1991). Summarizing, various studies using radiolabelled histamine H_3-receptor agonists provided evidence for coupling of the histamine H_3-receptor to a G-protein. However, the binding characteristics of these agonists are complex and results obtained by several authors appear to be to a certain extent controversial.

The affinities of histamine H_3-receptor antagonists largely correlate to the antagonistic activities obtained from functional studies (Table 4). With respect to histamine H_3-receptor agonists, the agonistic activities are closely related to their high affinity binding sites (Table 4). In contrast, in binding studies using radiolabelled agonists the affinities of histamine H_3-receptor agonists reported are approximately 10-fold higher than their corresponding pD_2-values. Hence, the pK_D-values observed in these receptor binding studies may not reflect their affinity for the functional histamine H_3-receptor.

A detailed description of the distribution of histamine H_3-receptors in rat brain has been published using [^{3}H](R)α-methylhistamine as a radioligand (Pollard *et al.*, 1993). The distribution of [^{125}I]iodophenpropit binding sites was essentially the same as for [^{3}H](R)α-methylhistamine, as observed by Pollard and colleagues. Histamine H_3-receptors showed a clearly distinct distribution compared to histamine H_1- and H_2-receptors (Garbarg *et al.*, 1992). Moreover, the distribution of histamine H_3-receptors appeared to be distinct from the distribution of histaminergic nerve endings in rat brain (Watanabe *et al.*, 1984). This observation may be explained by the occurrence of the

Table 4. *The affinity of histamine H_3-receptor agonists and antagonists towards [^{125}I]iodophenpropit binding sites on rat cerebral cortex compared with their functional potencies.*

H_3-agonists:	pK_D		pD_2
histamine	7.4		7.4 [c]
(R)α-methylhistamine	8.5		8.4 [c]
(S)α-methylhistamine	6.6		6.3 [c]
imetit	8.7		9.3 [c]
immepip	8.6		8.0 [d]
H_3-antagonists:	pK_D		pA_2
clobenpropit	9.0		9.9 [a]
iodophenpropit	9.0		9.6 [b]
thioperamide	8.4		8.5 [a]
impromidine	7.3		7.2 [c]
	($pK_{D,1}$)	($pK_{D,2}$)	
burimamide	7.7	6.1	7.2 [c]
dimaprit	6.4	4.4	5.5 [c]

The affinities of the various ligands towards the [^{125}I]iodophenpropit binding sites are expressed as -log K_D (= pK_D). The pK_D-value for agonists correspond to their high affinity binding site. The functional potencies of H_3-receptor agonists and antagonists are expressed as pD_2- and pA_2-values respectively. [a]Determined on guinea-pig intestine (Van der Goot *et al.*, 1992); [b]Determined on guinea-pig intestine (Jansen *et al.*, 1992); [c]Determined on rat cerebral cortex (Leurs *et al.*, 1992); [d]Determined on guinea-pig jejunum (Vollinga *et al.*, 1994).

H_3-receptor as a heteroreceptor. The histamine H_3-receptor was first characterized as an autoreceptor inhibiting both synthesis and release of neuronal histamine (Arrang *et al.*, 1983; Van der Werf *et al.*, 1987). However, several studies have indicated the involvement of histamine H_3-receptors in modulation of the release of other neurotransmitters such as serotonin (Fink *et al.*, 1990), noradrenaline (Schlicker *et al.*, 1989) and acetylcholine (Clapham & Kilpatrick, 1992; Vollinga *et al.*, 1992; Arrang *et al.*, 1995). Also, H_3-receptors may be localized postsynaptically (see Chapter 6).

In conclusion, [^{125}I]iodophenpropit binds to rat cerebral cortex membranes with a high affinity, saturability and reversibility. [^{125}I]Iodophenpropit binding sites in rat brain are likely to represent histamine H_3-receptors as the binding is displaced by selective histamine H_3-receptor ligands, in contrast to ligands selective for other receptors. The biphasic displacement of [^{125}I]iodophenpropit by burimamide and dimaprit may be indicative for the existence of histamine H_3-receptor subtypes. The sensitivity of

histamine H_3-receptor agonist competition binding curves to guanine nucleotides confirms the results of previous experiments indicating the interaction of histamine H_3-receptors with G-proteins. [125I]Iodophenpropit binding sites are heterogeneously distributed in rat brain and show a distribution pattern essentially the same as previously reported for the H_3-agonist [3H](R)α-methylhistamine. [125I]Iodophenpropit is a valuable new tool to study histamine H_3-receptors.

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- *Chapter 4* -

Characterization of histamine H_3-receptors in mouse brain using the H_3-antagonist [^{125}I]-iodophenpropit; an *in vitro* study

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Abstract

We have characterized the binding of the histamine H_3-receptor antagonist [^{125}I]-iodophenpropit to mouse brain. [^{125}I]-Iodophenpropit saturably bound to mouse brain membranes with a dissociation constant of 0.49 ± 0.04 nM and a receptor binding density of 290 ± 8 fmol·mg^{-1} of protein. Saturation binding analysis revealed binding of [^{125}I]-iodophenpropit to a single class of sites, showing linear Scatchard plots and Hill coefficients not different from unity (n_H = 0.98 ± 0.02). At a concentration of 0.3 nM [^{125}I]-iodophenpropit, specific binding represented about 75% of the total binding. Competition binding curves for H_3-receptor antagonists were fitted best to a one site model, showing K_D-values in general accordance with the pA_2-values obtained in mouse cerebral cortex. Displacement of [^{125}I]-iodophenpropit by the H_3-receptor agonists (R)α-methylhistamine, immepip, imetit and histamine were fitted best to a two site model. Competition binding curves of (R)α-methylhistamine showed a rightward shift upon incubation with GTPγS (10 μM) indicating the involvement of G-proteins in H_3-agonist binding. In contrast, competition binding curves of the antagonists iodophenpropit, thioperamide and burimamide were not affected by GTPγS (10 μM). Autoradiographic experiments showed that [^{125}I]-iodophenpropit binding sites were heterogeneously distributed, similarly to the distribution of histamine H_3-receptors reported in rat brain (Jansen *et al.*, 1994). Highest densities were observed in the cerebral cortex, the caudate putamen, the nucleus accumbens, the globus pallidus and the substantia nigra.

In conclusion, we have demonstrated that in mouse brain, [^{125}I]-iodophenpropit selectively binds to histamine H_3-receptors. We also observed that the mouse brain H_3-receptors labelled by [^{125}I]-iodophenpropit displayed binding characteristics and a distribution similar to rat brain.

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Introduction

The histamine H_3-receptor has been well documented as a presynaptic receptor modulating neuronal histamine release in the CNS and the release of other neurotransmitters, both in CNS and PNS (Schwartz *et al.*, 1991; Leurs & Timmerman, 1992; Schlicker *et al.*, 1994). Histamine H_3-receptors have been identified in different mammalian species, from rodents to primates. Activation of H_3-receptors in the CNS has been demonstrated to modulate sleep and wakefulness, cognition, locomotion, feeding behaviour and electrically induced convulsions (for review see Schwartz *et al.*, 1991 and Onodera *et al.*, 1994). The molecular pharmacological mechanisms underlying these effects are likely related to modulation of the release of different neurotransmitters like histamine (H_3-autoreceptors, Arrang *et al.*, 1983; Van der Werf *et al.*, 1987), serotonin (Fink *et al.*, 1990; Alves-Rodrigues *et al.*, 1995), noradrenaline (Schlicker *et al.*, 1989), acetylcholine (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995) and neuropeptides (Matsubara *et al.*, 1992). Histamine H_3-receptors may also attain their effects via yet unidentified postsynaptic mechanisms (Cumming *et al.*, 1991; Ryu *et al.*, 1994).

Histamine H_3-receptors have been characterized by receptor binding studies performed in rat (Arrang *et al.*, 1990; West *et al.*, 1990a; Jansen *et al.*, 1994; Ligneau *et al.*, 1994), guinea pig (Korte *et al.*, 1990; Kilpatrick & Michel, 1991) and, to a less extent, in bovine (Zweig *et al.*, 1992) and primate brain (Martinez-Mir *et al.*, 1990). Characterization of H_3-receptors in mouse brain is based almost exclusively on functional studies (Kathmann *et al.*, 1993; Leurs *et al.*, 1996). The mouse has been frequently used to study the pharmacology of CNS H_3-receptors *in vivo*, using the H_3-agonist (R)α-methylhistamine and the H_3-antagonist thioperamide (Schwartz *et al.*, 1991; Onodera *et al.*, 1994). The *in vivo* potencies of both ligands in the mouse were consistent with their potencies reported in other species. The H_3-antagonist clobenpropit however, was reported to inhibit electrically induced convulsions in mice (Yokoyama *et al.*, 1994) at doses about ten fold lower than the doses required to observe CNS effects in rats (Barnes *et al.*, 1993). At present it is not clear whether this difference is due to a species difference or to H_3-receptor heterogeneity.

The distribution of binding sites of the H_3-agonist [^{3}H]N$^\alpha$-methylhistamine in mouse forebrain has been visualized with autoradiography (Cumming *et al.*, 1994). A pharmacological characterization of the [^{3}H]N$^\alpha$-methylhistamine binding sites using different selective ligands was not performed however. The present study was conducted to provide a more detailed characterization of cerebral histamine H_3-receptors in mouse brain. We have used the recently introduced H_3-antagonist [^{125}I]-iodophenpropit as a

radioligand (Jansen *et al.*, 1992; Jansen *et al.*, 1994). The affinities of different ligands for [^{125}I]-iodophenpropit binding sites were determined in mouse brain membrane preparations. Moreover, we examined the distribution of [^{125}I]-iodophenpropit binding sites in mouse CNS using receptor autoradiography.

Materials and methods

Preparation of mouse cerebral membranes and receptor binding studies

Balb/c mice (20-25 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were rapidly removed. The brains were homogenized in 15 volumes (v/w) ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) using a glass-teflon homogenizer. Subsequently membrane fractions were prepared as previously described (Jansen *et al.*, 1994). Receptor binding experiments were performed at 37°C in the Tris-HCl buffer (pH 7.4 at 37°C) with a total incubation volume of 0.5 ml. Determinations were performed in triplicate. In saturation experiments membranes were incubated with [^{125}I]iodophenpropit in final concentrations ranging from 0.025 to 3 nM. In competition binding experiments a concentration of 0.25 nM [^{125}I]iodophenpropit was used. There was no significant difference between [^{125}I]iodophenpropit binding displaced by (R)α-methylhistamine (10 μM; 73±2% of total binding) and by thioperamide (0.3 μM; 77±3% of total binding, P>0.05; two tailed unpaired Student's t-test). H_3-Receptor agonists and H_3-receptor antagonists displaced [^{125}I]-iodophenpropit to the same level. Membrane suspensions were incubated with [^{125}I]iodophenpropit (Menge *et al.*, 1992) for 60 minutes to reach equilibrium. Incubations were started by the addition of 100 μl membranes (20 - 60 μg of protein per tube) and were terminated by adding 2 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°C) immediately followed by filtration through Whatman GF/C filters using a Brandel filtration apparatus. The radioactivity bound to the filters was measured by an LKB gamma counter. Protein concentrations were determined using the Bio-Rad Protein Assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard.

Data analysis and statistical evaluation

Dissociation constants for H_3-ligands in the absence and presence of GTPγS were compared using a two tailed unpaired Student's t-test. Saturation and competition binding experiments were evaluated using the non-linear curve fitting program LIGAND (Munson & Rodbard, 1980) on a Macintosh computer. Binding curves were fitted (unweighed) to a one and a two site model respectively, and statistically tested on the increasing goodness of the fit for a model with additional parameters, using a probability level of 5%.

Receptor autoradiographic experiments

Balb/c mice (20-25 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were rapidly removed. Brains were frozen in isopentane at -40°C. Cryostate sections (transversal or sagittal, 14 μm) were cut, mounted onto gelatin/chromalum-coated glass slides and were stored at -80°C until use. For the autoradiographic experiments, tissue sections were thawed and were incubated with 0.3 nM [^{125}I]-iodophenpropit for 60 minutes at 37°C in 50 mM Tris-HCl buffer containing 145 mM NaCl, 5 mM $MgCl_2$ and 0.25% BSA (pH 7.4 at 37°C). Non-specific binding was determined by incubation of adjacent sections in the presence of 1 μM (R)α-methylhistamine. To stop the incubations the sections were rinsed once with ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) and were subsequently washed twice for 15 minutes in ice-cold Tris-HCl buffer and 15 seconds in ice-cold distilled water. Sections were dried by a stream of cold air and were exposed to Hyperfilm (Amersham International, U.K.) for 20 hours.

Chemicals

[^{125}I]-Iodophenpropit (Radionuclide Center, Leiden/Amsterdam Center for Drug Research, Vrije Universiteit, Amsterdam) was labelled to a specific activity of 1900 Ci $mmol^{-1}$ as described by Menge *et al.* (1992). The following drugs were used: iodophenpropit dihydrobromide (laboratory stock), clobenpropit dihydrobromide (laboratory stock), thioperamide maleate (laboratory stock), iodoproxyfan (gift from Prof.Dr. W. Schunack, Berlin, Germany), burimamide (gift from SK&F Laboratories, UK), impromidine trihydrochloride (gift from SK&F Laboratories), dimaprit dihydrochloride (gift from SK&F Laboratories), histamine dihydrochloride (Sigma, St. Louis, USA), betahistine dihydrochloride (gift from Solvay Duphar, Netherlands), ondansetron (gift from Solvay Duphar), (R)α-methylhistamine dihydrochloride (Research Biochemicals International, Natick, USA), (S)α-methylhistamine dihydrobromide (Tocris Cookson, St. Louis, USA), imetit dihydrobromide (VUF8325, laboratory stock), immepip dihydrobromide (VUF4708; 4-(1*H*-imidazol-4-ylmethyl)piperidine dihydrobromide, laboratory stock), mepyramine hydrochloride (Sigma), tiotidine (ICI, Macclesfield, U.K.), yohimbine (Roth KG, Karlsruhe, Germany), guanosine 5'-O-(3-thio)triphosphate (GTPγS; Sigma), Bovine serum albumin (BSA; Sigma) and polyethylenimine (Aldrich, Zwijndrecht, Netherlands). Ligands (solutions of 1-10 mM) were dissolved in distilled water or in Tris-HCl buffer and were further diluted in Tris-HCl buffer. Ondansetron (1 mM) and haloperidol (10 mM) were dissolved in DMSO and ethanol, respectively.

Results

Binding of [125I]-iodophenpropit to mouse brain membranes was saturable and of high affinity (K_D = 0.49 ± 0.04 nM; B_{max} = 290 ± 8 fmol·mg^{-1} of protein; n =3; Figure 1). Saturation binding curves yielded linear Scatchard plots (not shown) and Hill-coefficients not different from unity (n_H = 0.98 ± 0.02). The nonspecific binding as determined using thioperamide (0.3 μM) increased linearly with the concentration of [125I]-iodophenpropit (Figure 1). At a concentration of 0.3 nM of [125I]-iodophenpropit the specific binding represented 77 ± 3% of the total binding. Under the same experimental conditions 73 ± 2% of the [125I]-iodophenpropit binding was displaced by (R)-α-methylhistamine (10 μM).

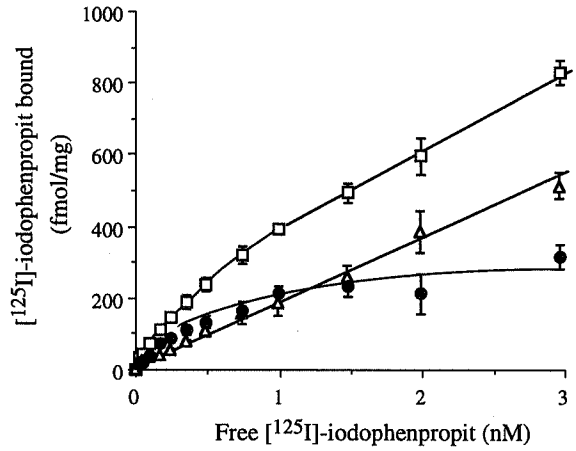

Figure 1. *Saturation binding of [125I]-iodophenpropit to mouse brain membranes. Symbols: (□), total binding; (Δ), nonspecific binding; (●), specific binding. Nonspecific binding was determined in the presence of thioperamide (0.3 μM). A representative experiment is shown. Each experiment was performed with triplicate determinations. Values are represented as the mean ± s.d. Membrane suspensions were incubated with [125I]-iodophenpropit (0.03 - 3 nM) for one hour as described in 'materials and methods'.*

Competition binding curves of H_3-receptor antagonists were fitted best to a one site model (P>0.05; Figure 2A, Table 1). For the H_3-antagonists the following rank order of potency was obtained: clobenpropit, iodophenpropit > iodoproxyfan > thioperamide > impromidine > burimamide > dimaprit > betahistine. GTPγS (10μM) did not significantly affect competition binding curves of the antagonists iodophenpropit (K_D(-GTPγS) = 0.6 ± 0.3 nM; K_D(+GTPγS) = 0.7 ± 0.2 nM, n=4; Figure 2C), thioperamide (K_D(-GTPγS)

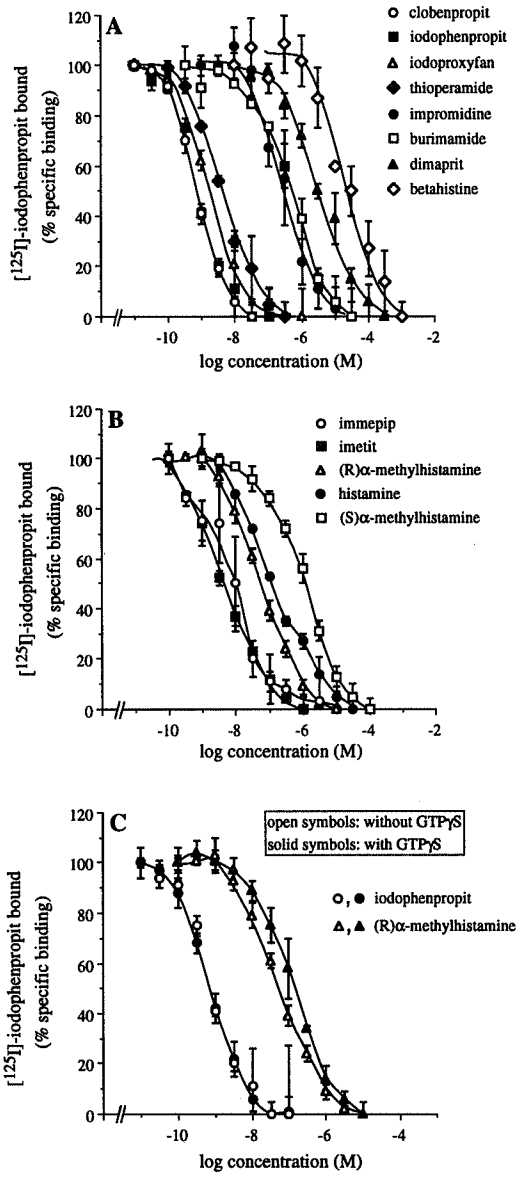

Figure 2. *Displacement of [^{125}I]-iodophenpropit (0.25 nM) binding to mouse brain membranes. Competition binding curves of H_3-antagonists (**A**) and H_3-agonists (**B**). Sensitivity of competition binding curves of (R)α-methylhistamine and of iodophenpropit towards GTPγS (10 μM) (**C**). Values are given as the mean ± V.C. of representative experiments. The specific binding, defined as the difference between total binding and binding in the presence of 0.3 μM thioperamide or (R)-α-methylhistamine, represented about 77 ± 3% and 73 ± 2% of the total binding, respectively. Membrane suspensions were incubated with [^{125}I]-iodophenpropit (0.25 nM) for one hour as described in 'materials and methods'.*

Table 1. *Dissociation constants of histamine H_3-receptor antagonists and of other ligands for [125I]-iodophenpropit binding sites on mouse whole brain.*

ligand	K_D (nM)	n
H_3-antagonists:		
clobenpropit	0.6 ± 0.2	3
iodophenpropit	0.6 ± 0.3	4
iodoproxyfan	1.5 ± 0.5	4
thioperamide	4.4 ± 1.2	11
impromidine	174 ± 23	3
burimamide	454 ± 283	4
dimaprit	2,800 ± 1,000	4
betahistine	19,000 ± 2,000	3
other ligands:		
mepyramine	> 10,000	3
tiotidine	> 10,000	3
ondansetron	> 10,000	3
yohimbine	> 10,000	3
haloperidol	6,100 ± 1,600	3

All compounds displayed were best fit according to a one-site model ($P > 0.05$). For burimamide and dimaprit in one of four experiments, competition binding curves were fitted best to a two site model (P-values of 0.046 and 0.020, respectively. Values are given as the mean ± s.d. (n: number of experiments).

= 3.8 ± 0.2 nM; K_D(+GTPγS) = 3.0 ± 0.4 nM, n=3) and burimamide (K_D(-GTPγS) = 580 ± 336 nM; K_D(+GTPγS) = 378 ± 122 nM, n=3).

Displacement of [125I]-iodophenpropit by the H_3-agonists (R)α-methylhistamine, imetit, immepip and histamine was fitted best to a two site model ($P<0.05$; Figure 2B, Table 2). Competition binding curves of (S)α-methylhistamine were significantly fitted best to a one site model (Table 2). A more than ten-fold difference in affinity between (R)α-methylhistamine and (S)α-methylhistamine for [125I]-iodophenpropit binding sites was found (Figure 2B), consistent with the stereoselectivity of the enantiomers reported in functional studies (Arrang *et al.*, 1987a). Competition binding curves of (R)α-methylhistamine showed a significant rightward shift upon incubation with the guanine nucleotide GTPγS (10 μM; Figure 2C).

The histamine H_1-receptor antagonist mepyramine and the H_2-receptor antagonist tiotidine showed a low affinity (K_D > 10 μM) towards [125I]-iodophenpropit binding sites (Table

1). In a previous screening, [^{125}I]-iodophenpropit was found to exhibit a high to moderate affinity for 5-HT$_3$ (11 nM), α_2 (120 nM), and σ-receptors (170 nM; Leurs *et al.*, 1995). In our study, ligands selective for these receptors i.e. ondansetron (5-HT$_3$), yohimbine (α_2) and haloperidol (σ) showed dissociation constants for [^{125}I]-iodophenpropit binding sites above 1 µM (Table 1).

Table 2. *Dissociation constants of histamine H_3-receptor agonists for [^{125}I]-iodophenpropit binding sites on mouse whole brain.*

	high affinity		*low affinity*	
agonist:	K_H (nM)	N(%)	K_L (nM)	N(%)
histamine	17 ± 15	45 ± 21	1,500 ± 1,500	55 ± 21
(R)α-methylhistamine	12 ± 3	61 ± 3	233 ± 96	39 ± 3
" + 10 µM GTPγS	26 ± 7*	53 ± 10	537 ± 234	47 ± 10
(S)α-methylhistamine	-	-	1,200 ± 100	100**
imetit	0.8 ± 0.4	62 ± 4	215 ± 324	38 ± 4
immepip	0.6 ± 0.7	48 ± 18	41 ± 28	52 ± 18

Values are given as the mean ± s.d. of four experiments. *Significantly different from K_H-value in the absence of GTPγS ($P < 0.05$); **Two site model was not significantly better than the one site model ($P > 0.05$ in two of four experiments). N(%): percentage of the total specific binding of the corresponding binding site.

Autoradiograms showed that [^{125}I]-iodophenpropit binding sites were heterogeneously distributed in mouse brain (Figure 3). Highest densities were observed in the cerebral cortex, the caudate putamen, the nucleus accumbens, the globus pallidus and the substantia nigra. Moderate densities of [^{125}I]-iodophenpropit binding sites were observed in the thalamus, the hypothalamus and the hippocampus. The cerebellum and the brain stem showed low binding densities. Nonspecific binding was homogeneously distributed in all of the brain areas examined (Figure 3).

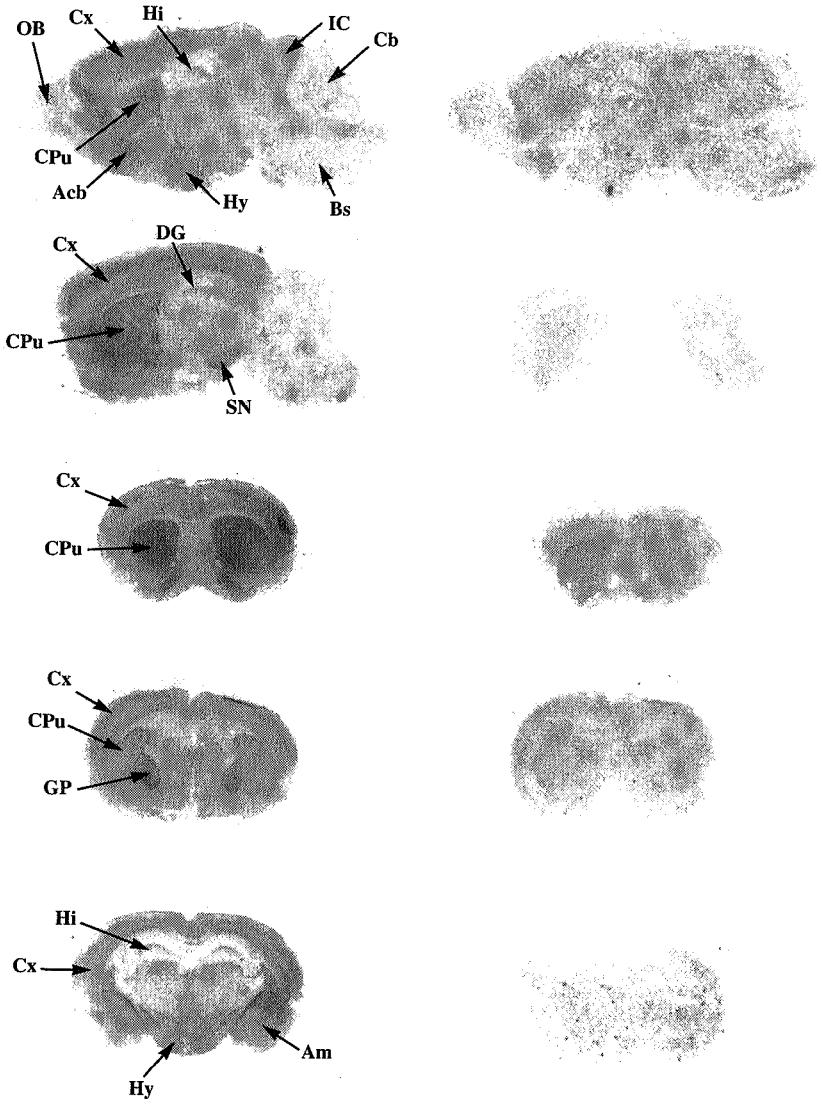

Figure 3. *Autoradiograms of [125I]-iodophenpropit binding to rat brain sections. Left column: total binding of [125I]-iodophenpropit (0.3 nM); right column: binding in the presence of (R)α-methylhistamine (1 μM). Sections (14 μm) were incubated with [125I]-iodophenpropit (0.3 nM) for one hour as described in 'materials and methods'. Abbreviations: Acb, accumbens; Am, amygdala; Bs, brain stem; Cb, cerebellum; CPu, caudate putamen; Cx, cortex; DG, dentate gyrus; GP, globus pallidus; Hi, hippocampus; Hy, hypothalamus; IC, inferior colliculus; OB, olfactory bulb; SN, substantia nigra.*

Discussion

The histamine H_3-receptor was initially identified as the autoreceptor of histaminergic neurons in the brain (Arrang *et al.*, 1983). Activation of the H_3-receptor resulted in an inhibition of histamine release (Arrang *et al.*, 1983; Van der Werf *et al.*, 1987) and synthesis (Arrang, 1987b) in rat brain. From studies measuring neurotransmitter release from rat brain slices H_3-receptors were demonstrated to modulate the release of other transmitters like noradrenaline (Schlicker *et al.*, 1989), serotonin (Fink *et al.*, 1990; Alves-Rodrigues *et al.*, 1995), dopamine (Schlicker *et al.*, 1993), acetylcholine (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995) and neuropeptides (Matsubara *et al.*, 1992). The modulation of neurotransmitter release is a conceivable mechanism underlying the CNS effects of H_3-ligands *in vivo*.

The mouse is a frequently used species for *in vitro* and *in vivo* studies on H_3-receptor function. Activation of histamine H_3-receptors has been reported to cause an inhibition of electrically induced release of noradrenaline (Schlicker *et al.*, 1989) from mouse cerebral cortex slices *in vitro*. The histamine H_3-receptor mediated inhibition of noradrenaline release has been used as a model system to determine the activity of potential new H_3-receptor ligands (Kathmann *et al.*, 1993; Leurs *et al.*, 1996). From *in vivo* studies, the activation of mouse brain H_3-receptors was demonstrated to affect feeding (Oishi *et al.*, 1987), cognition (Meguro *et al.*, 1995), anxiety (Imaizumi & Onodera, 1993), locomotion (Sakai *et al.*, 1991; Clapham & Kilpatrick, 1994) and convulsions (Yokoyama *et al.*, 1993; Yokoyama *et al.*, 1994). Despite various reports on functional studies on mouse histamine H_3-receptors, little information has been presented on the pharmacological characteristics of these receptors with receptor binding studies. In one report the H_3-agonist [^{3}H]N$^{\alpha}$-methylhistamine has been used to study the distribution of H_3-receptors by means of autoradiography (Cumming *et al.*, 1994). Binding sites of the radiolabelled agonist showed a heterogeneous distribution which was essentially the same as for rat and for guinea pig brain. A pharmacological characterization of the [^{3}H]N$^{\alpha}$-methylhistamine binding sites using different selective ligands was not performed in this study however. In the present report we describe the pharmacological characterization of histamine H_3-receptor binding sites in mouse brain, using a radiolabelled antagonist.

[^{125}I]-Iodophenpropit has been successfully introduced as a tool for H_3-receptor binding studies in rat brain (Jansen *et al.*, 1992; Jansen *et al.*, 1994). Binding of [^{125}I]-iodophenpropit to membranes of rat cerebral cortex was of high affinity, saturable and reversible in nature. Saturation binding experiments performed on mouse whole brain membranes revealed binding of [^{125}I]-iodophenpropit to a single site with a K_D-value in accordance with a K_D-value found in rat cerebral cortex slices (Table 3). Affinities of

both, H_3-antagonists and H_3-agonists, were in general accordance with their functional potencies as determined in mouse brain (Table 3). The sensitivity of competition binding curves of the H_3-agonist (R)α-methylhistamine to GTPγS is in line with the previously indicated interaction of H_3-receptors with G-proteins (Arrang *et al.*, 1990; West *et al.*, 1990b; Jansen *et al.*, 1994; Clark & Hill, 1996). The receptor autoradiographic studies revealed that the distribution of [^{125}I]-iodophenpropit binding sites in mouse brain was comparable to the distribution of [^{125}I]-iodophenpropit binding sites in the rat (Jansen *et al.*, 1994) and to the distribution of radiolabelled agonist binding sites described in rat

Table 3. *Affinities of histamine H_3-receptor agonists and antagonists for [^{125}I]-iodophenpropit binding sites in mouse brain. A comparison with their functional potencies and with their binding affinities in rat cerebral cortex.*

compounds	mouse brain		rat cerebral cortex[1]
	pK_D	pA_2	pK_D
H_3-antagonists:			
clobenpropit	9.3	9.6[a]	9.0
iodophenpropit	9.2	-	9.0
iodoproxyfan	8.8	9.0[b]	8.6
thioperamide	8.4	8.4[c]	8.4
impromidine	6.8	6.9[c]	7.3
betahistine	4.7	-	< 5
burimamide	6.3	7.1[c]	8.0, 6.6
dimaprit	5.6	6.2[a]	6.4, 4.4
	pK_D[2]	pD_2	pK_D[2]
H_3-agonists:			
histamine	7.8	7.0[a]	7.4
(R)α-methylhistamine	7.9	7.8[a]	8.5
(S)α-methylhistamine	5.9	5.7[d]	6.6
imetit	9.1	8.9[a]	8.7
immepip	9.2	9.3[e]	8.6

Receptor binding affinities are expressed as the -logK_D (pK_D). pD_2- and pA_2-Values correspond to the H_3-receptor mediated inhibition of [^{3}H]noradrenaline release from mouse cerebral cortex slices. [1]Values from Jansen *et al.*, 1994. [2]The pK_D-values for agonists correspond to their high affinity binding site, except for (S)α-methylhistamine (two site fit not significantly better than one site fit, P>0.05). [a]Kathmann *et al.*, 1993; [b]Schlicker *et al.*, 1996; [c]Schlicker *et al.*, 1989; [d]Leurs *et al.*, 1996; [e]Alves-Rodrigues, 1996.

(Cumming *et al.*, 1991; Pollard *et al.*, 1993), mouse (Cumming *et al.*, 1994) and guinea-pig brain (Cumming *et al.*, 1994). In mouse brain, a relatively high [^{125}I]-iodophenpropit binding was seen in the globus pallidus as compared to rat brain. Minor species differences have also been reported between mouse and guinea-pig (Cumming *et al.*, 1994). [^{3}H]N$^{\alpha}$-Methylhistamine binding in the guinea-pig insular cortex exceeded binding in the striatum, whereas in the mouse the ratio between the two brain areas was reverse (Cumming *et al.*, 1994). Although there may be small species differences between rat, mouse and guinea-pig, the overall distribution of histamine H_3-receptor binding sites in these species is the same. With this respect, cerebral histamine H_3-receptors differ from H_1- and H_2-receptors displaying considerable distributional differences between species (Garbarg *et al.*, 1992).

Recently, the affinity of iodophenpropit for a variety of different receptors has been determined (Leurs *et al.*, 1995). Iodophenpropit displayed a high to moderate affinity towards 5-HT_3-serotonergic (11 nM), α_2-adrenergic (120 nM), and σ-receptors (170 nM). Ligands selective for these receptors showed a low affinity for [^{125}I]-iodophenpropit binding sites on mouse brain homogenates, indicating that binding of [^{125}I]-iodophenpropit to these receptors was not observed at the experimental conditions used. Receptor binding studies using the radiolabelled H_3-antagonists [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994), [^{3}H]-thioperamide (Alves-Rodrigues *et al.*, 1996) and [^{125}I]-iodophenpropit (see Chapter 5) indicated that these ligands show a relatively high affinity towards non-H_3-receptor components, in rat brain. This phenomenon was apparent from the observation that, in specific brain areas, the total radioligand binding displaceable by H_3-antagonists exceeded the total radioligand binding displaceable by H_3-agonists (Ligneau *et al.*, 1994; Alves-Rodrigues *et al.*, 1996). Thus, the definition of the nonspecific binding of these radioligands needs critical evaluation. In rats, for [^{125}I]-iodophenpropit, depending on the rat brain area used, 8% to 40% of the total binding displaced by antagonists was not displaced by agonists (see Chapter 5). In mouse whole brain membranes, binding displaced by (R)α-methylhistamine was not significantly different from binding displaced by thioperamide, indicating that the non-H_3-receptor component as observed in rats is not detected in mouse brain. Moreover, it is noteworthy that the specific [^{125}I]-iodophenpropit binding observed in mouse brain membranes (~75% of total binding) was higher as compared to rat cerebral cortex (50-60% of total binding; (Jansen *et al.*, 1994).

Although in mouse brain homogenates the receptor binding affinities of most ligands closely parallelled the affinities found in rat brain, some minor differences between the two species were observed as well (Table 3). Previously, we have reported on the biphasic displacement of [^{125}I]-iodophenpropit from rat cerebral cortex membranes by

burimamide and by dimaprit (Jansen *et al.*, 1994). A biphasic displacement by these ligands was not observed in mouse whole brain homogenates. These discrepancies might reflect a species difference. However, the differences may also result from the different tissue preparation used (i.e. mouse whole brain versus rat cerebral cortex). Biphasic competition binding curves of burimamide (Arrang *et al.*, 1990; West *et al.*, 1990b) and of thioperamide (West *et al.*, 1990b) have previously been reported in rat brain using radiolabelled agonists. From the biphasic displacement the existence of H_3-receptor subtypes (H_{3A} and H_{3B}) was proposed (West *et al.*, 1990b). It should be noted however that reports on the biphasic displacement were not always consistent (West *et al.*, 1990a; Kilpatrick & Michel, 1991; Clark & Hill, 1995). The discrepancies might in part result from the relatively small differences in affinities between the two binding sites in rats. Hence, this could also explain the monophasic nature of competition binding curves for burimamide and dimaprit in the mouse brain as observed in the present study.

In vivo studies provided evidence for a possible species difference between rat and mouse, based on the studie with the H_3-antagonist clobenpropit. In rats, (R)α-methylhistamine induced water consumption (Barnes *et al.*, 1993) and neuronal histamine release (see Chapter 8) were modulated by clobenpropit at doses of 10 to 15 mg/kg. In mice however, at about ten fold lower doses (~ 1mg/kg) clobenpropit antagonized electrically induced convulsions, decreased central histamine levels and increased brain histidine decarboxylase activity (Yokoyama *et al.*, 1994). The difference in potency of clobenpropit between rat and mouse may result from (a combination of) several mechanisms i.e. a species difference in pharmacokinetics of the compound, a species difference of H_3-receptors and/or the existence of H_3-receptor subtypes. We recently investigated the brain penetration of clobenpropit in rats and mice by *ex vivo* receptor binding. In both species, the *ex vivo* displacement of [^{125}I]-iodophenpropit by clobenpropit was observed at the same dose range however (Mochizuki *et al.*, 1996). Additionally, in the present study the affinity of clobenpropit for [^{125}I]-iodophenpropit binding sites in mouse (K_D = 0.6 ± 0.2 nM) was not different from the affinity in rat cerebral cortex (K_D = 0.9 ± 0.4 nM, see Chapter 3). Altogether, these results do not explain the differential effects of clobenpropit in rats and mice from a pharmacokinetic or a pharmacodynamic difference. Yet, a satisfactory explanation for the differential potency of clobenpropit between rats and mice cannot be given.

In conclusion, in the present study we have demonstrated that [^{125}I]-iodophenpropit bound with high affinity and saturably to mouse whole brain membranes. The affinities of H_3-receptor agonists and antagonists for the [^{125}I]-iodophenpropit binding sites were in accordance with the functional potencies on the H_3-receptor mediated modulation of electrically evoked noradrenaline release, indicating that the [^{125}I]-iodophenpropit binding

sites represented functional H_3-receptor binding sites. Autoradiographic studies demonstrated that [^{125}I]-iodophenpropit binding sites were distributed heterogeneously, showing a general overlap with the distribution of histamine H_3-receptor binding sites observed in the rat. [^{125}I]-Iodophenpropit can be regarded as a reliable radioligand to study histamine H_3-receptors in mouse brain.

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- *Chapter 5* -

The distribution of binding sites of the histamine H_3-receptor antagonist [^{125}I]iodophenpropit in rat brain studied by storage phosphor autoradiography; chemically unrelated H_3-antagonists reveal a homogenous population of binding sites

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Abstract

We have further characterized the binding sites of the histamine H_3-receptor antagonist [^{125}I]iodophenpropit in the CNS of the rat using autoradiography. Radiolabelled sections of different rat brain regions were quantified applying the storage phosphor imaging technique. [^{125}I]Iodophenpropit binding sites showed a heterogeneous distribution. Highest densities were found in the cerebral cortex, the striatum and the substantia nigra. In order to study a possible heterogeneity of histamine H_3-receptors, [^{125}I]iodophenpropit binding sites were characterized in different rat brain areas i.e. cortical areas, caudate putamen, nucleus accumbens, hippocampus, thalamus, hypothalamus and substantia nigra. Displacement studies of [^{125}I]iodophenpropit using a chemically heterogeneous group of H_3-antagonists did not reveal a heterogeneity of [^{125}I]iodophenpropit binding sites in the above mentioned brain areas. Affinities of the histaminergic ligands determined in the autoradiographic studies related well to their functional potencies described in literature so far. In some brain areas, a difference was found between [^{125}I]iodophenpropit binding displaceable by agonists and by antagonists which has implications for the definition of the specific binding in these regions.

In conclusion, we have shown that [^{125}I]iodophenpropit is a suitable radioligand for the characterization of histamine H_3-receptors in rat brain. [^{125}I]Iodophenpropit binding sites in different brain areas were not discriminated by a chemically heterogeneous group of H_3-receptor antagonists.

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Introduction

The histamine H_3-receptor has been characterized as an auto- and a heteroreceptor regulating the synthesis and release of neuronal histamine in the CNS and the release of a variety of other neurotransmitters in the CNS and PNS (Schwartz *et al.*, 1991; Leurs & Timmerman, 1992; Schlicker *et al.*, 1994b). The H_3-receptor plays an important role mediating functions of neuronal histamine (for review see: Schwartz *et al.*, 1991; Onodera *et al.*, 1994) and in the interaction of histaminergic system with other neurotransmitter systems.

The pharmacology of histamine H_3-receptors has previously been studied in membrane preparations using the radiolabelled agonists [^{3}H]histamine, [^{3}H](R)α-methylhistamine and [^{3}H]N$^{\alpha}$-methylhistamine (review see: Leurs *et al.*, 1995b). In these studies evidence was provided for the coupling of the histamine H_3-receptor to G-proteins. Autoradiographic studies using radiolabelled agonists revealed that histamine H_3-receptors are widely distributed in rat CNS (Arrang *et al.*, 1987 ; Cumming *et al.*, 1991; Pollard *et al.*, 1993). There is some evidence for the existence of H_3-receptor subtypes based on the biphasic displacement of the tritiated agonists by burimamide and thioperamide (West *et al.*, 1990b). However, due to controversial reports on this matter the existence of subtypes has to be further explored.

One of the drawbacks of the use of radiolabelled histamine H_3-receptor agonists in receptor binding studies is that their binding dynamics are complicated as they include both, information on ligand-receptor interaction and ternary complex formation of the agonist with the receptor and G-proteins ("high affinity state"). Hence, radiolabelled antagonists are expected to constitute more reliable tools for receptor binding studies. We have previously introduced [^{125}I]iodophenpropit, a potent member of a group of isothiourea derived histamine H_3-receptor antagonists (Van der Goot *et al.*, 1992) as the first radiolabelled H_3-receptor antagonist (Menge *et al.*, 1992; Jansen *et al.*, 1992). [^{125}I]Iodophenpropit has been shown to be a suitable radioligand for *in vitro* characterization of H_3-receptors in rat cerebral cortex. The sensitivity to guanine nucleotides of [^{125}I]iodophenpropit displacement from rat cortical membrane preparations by agonists confirmed the interaction of H_3-receptors with G-proteins (Jansen *et al.*, 1994; Leurs *et al.*, 1996). Preliminary qualitative autoradiographic studies revealed that [^{125}I]iodophenpropit binding sites are heterogeneously distributed in rat CNS (Jansen *et al.*, 1994; see also Chapter 3). In addition to [^{125}I]iodophenpropit, more recently the H_3-receptor antagonists [^{125}I]iodoproxyfan (Ligneau *et al.*, 1994), [^{3}H]S-methylthioperamide (Yanai *et al.*, 1994), [^{3}H]thioperamide (Alves-Rodrigues *et al.*, 1996) and [^{3}H]GR168320 (Brown *et al.*, 1996) have been described as probes as well.

In the present study [^{125}I]iodophenpropit binding sites in rat CNS are further characterized using quantitative autoradiography. Performing binding studies on tissue sections allows the simultaneous determination of affinities in distinct brain areas located on the same section and has the additional advantage of a higher anatomical accuracy as compared to studies using membrane preparations. As a quantification method of the [^{125}I]labelled tissue sections we have used storage phosphor autoradiography, a new method which has several advantages compared to conventional methods used for computer assisted image analysis (Yanai *et al.*, 1992; Kanekal *et al.*, 1995; Tang *et al.*, 1995). We have therefore applied this procedure to determine the density of [^{125}I]iodophenpropit binding sites in various rat brain areas. To study a possible heterogeneity of histamine H_3-receptors in rat CNS, the affinities for the [^{125}I]iodophenpropit binding sites of a chemically heterogeneous series of histamine H_3-receptor antagonists were determined in ten different brain areas.

Materials and methods

Receptor autoradiographic procedure

Male Wistar rats (200-250 g, Harlan C.P.B., Zeist, The Netherlands) were decapitated. The brains were immediately removed and were frozen in isopentane at -40°C. Cryostate sections (transversal or sagittal, 14 μm) were cut, mounted onto gelatin/chromalum-coated glass slides and stored at -80°C until use. For receptor autoradiography, the tissue sections were thawed and incubated with 0.3 nM [^{125}I]iodophenpropit for 60 minutes at 37°C in 50 mM Tris-HCl buffer containing 145 mM NaCl, 5 mM $MgCl_2$ and 0.25% BSA (pH 7.4 at 37°C). To determine the densities of [^{125}I]iodophenpropit binding sites in different brain areas adjacent tissue sections were incubated (in triplicate or quadruplicate) with [^{125}I]iodophenpropit (0.3 nM) in the presence of either (R)α-methylhistamine (1 μM) or thioperamide (0.3 μM) to define the nonspecific binding. In displacement experiments transversal tissue sections were incubated (in duplicate) with [^{125}I]iodophenpropit (0.3 nM) in the presence of different concentrations of selected ligands. The tissue sections were randomized prior to the experiment to minimize the effect of (possible) rostro-caudal variation in receptor densities. To stop the incubations the sections were rinsed once with ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) and were subsequently washed twice for 15 minutes in ice-cold Tris-HCl buffer and 15 seconds in ice-cold distilled water. Sections were dried by a stream of cold air and were exposed to Hyperfilm (Amersham International, U.K.) for 20 hours and subsequently to a storage phosphor screen. Proper images were obtained already several hours after exposure. To increase the resolution of

the images the screens were exposed up to three days. Phosphor screens were scanned with a Phosphor Imager® (Molecular Dynamics, Sunnyvale, CA, USA) to obtain the digitized images. In some experiments, tissue sections were scraped from the slides with a razor blade in order to count the radioactivity by an LKB gamma counter.

Data analysis and statistical evaluation:
The storage phosphor images were analyzed and quantified using the program ImageQuant® (Molecular Dynamics). Brain areas of interest (100 to 600 pixels, pixel size 88 x 88 μm) were selected and the density was expressed as the average counts per pixel-value in the selected brain areas. Specific binding was calculated by substracting the nonspecific binding from the total binding. Binding displaced by (R)α-methylhistamine (1 μM) and thioperamide (0.3 μM) was compared using a two tailed unpaired Student's t-test. Differences were considered statistically significant at a probability level of 5%. Competition binding experiments were evaluated using the non-linear curve fitting program LIGAND (Munson & Rodbard, 1980) on a Macintosh computer. With the aid of this program binding curves were fitted (unweighed) to a one and a two site model respectively, and statistically tested on the increasing goodness of the fit for a model with additional parameters, based on the 'extra sum of squares' principle (Draper & Smith, 1966) using a probability level of 5%. Dissociation constants of H_3-ligands in different brain areas were compared using a two tailed, unpaired Student's t-tests with a Bonferroni correction.

Ligands
[^{125}I]Iodophenpropit (Radionuclide Center, Leiden/Amsterdam Center for Drug Research, Vrije Universiteit, Amsterdam) was labelled to a specific activity of 1900 Ci mmol^{-1} as described by Menge *et al.* (1992). The following drugs were used: iodophenpropit dihydrobromide (laboratory stock), thioperamide maleate (laboratory stock), iodoproxyfan (gift from Prof.Dr. W. Schunack, Berlin, Germany), burimamide (gift from SK&F Laboratories, UK), VUF4616 (N-(iso-propyl)-N'-[5-(4(5)-imidazolyl)pentyl]thiourea oxalate; laboratory stock), (R)α-methylhistamine dihydrochloride (Research Biochemicals International, Natick, USA), (S)α-methylhistamine dihydrobromide (Tocris Cookson, St. Louis, USA). Ligands (solutions of 1-10 mM) were dissolved in distilled water or in Tris-HCl buffer and were further diluted in Tris-HCl buffer.

Results

Storage phosphor imaging method

Autoradiograms of [^{125}I]iodophenpropit binding to sagittal sections of rat brain obtained after exposure to film and to storage phosphor screen are displayed in Figure 1. Both methods yield the same overall distribution pattern, but with respect to resolution, best images are obtained using film autoradiography, i.e. the laminar distribution in the cortex and hippocampus is not clearly observed in the phosphor autoradiographic image. Different brain areas were still clearly recognized on the phosphor autoradiographic image enabling the use of this method to quantify different brain regions. A linear relation between quantification with the phosphor imaging method compared to direct gamma counting was observed over a wide range of radioligand binding (Figure 2).

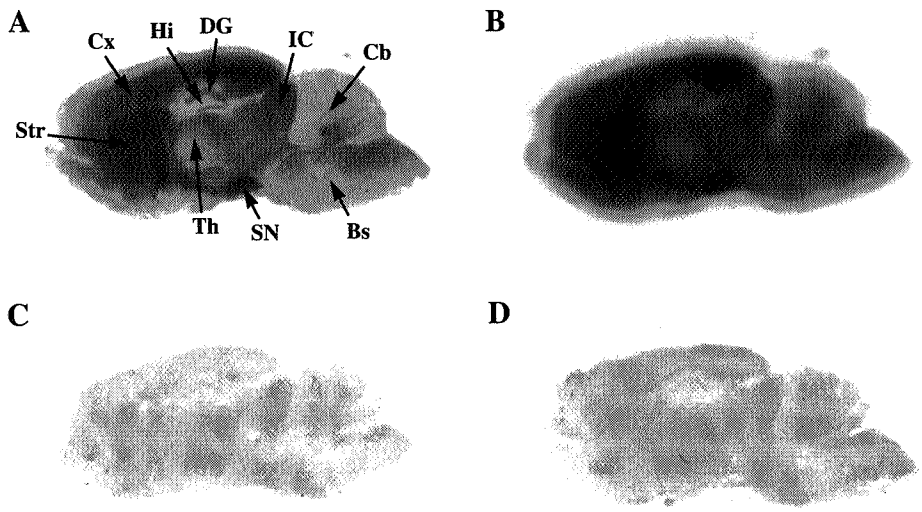

Figure 1. *Autoradiograms of [^{125}I]iodophenpropit binding to sagittal rat brain sections. (**A**, **C** and **D**) autoradiograms obtained from exposure to Hyperfilm. (**B**) the storage phosphor autoradiogram of panel A. (**A**) and (**B**): total binding; (**C**): nonspecific binding as determined with thioperamide (0.3 μM); (**D**): nonspecific binding as determined with (R)α-methylhistamine (1 μM). Tissue sections were incubated for one hour with [^{125}I]iodophenpropit (0.3 nM). Sections were exposed to Hyperfilm and storage phosphor screen, respectively (see 'materials and methods'). Representative autoradiograms are shown. Abbreviations: Bs, brain stem; Cb, cerebellum; Cx, cortex; DG, dentate gyrus; Hi, hippocampus; IC, inferior colliculus; SN, substantia nigra; Str, striatum; Th, thalamus.*

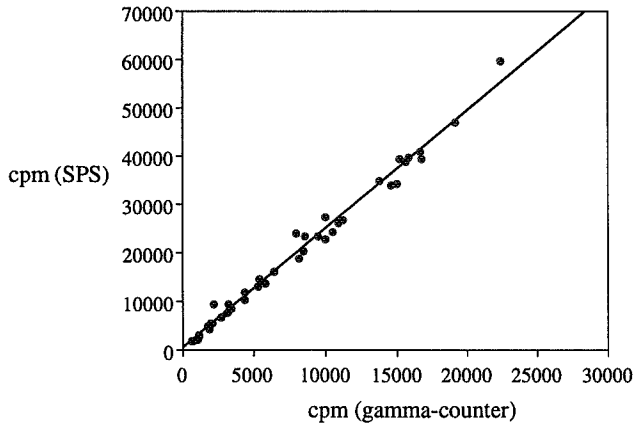

Figure 2. *Linear relation between quantification with storage phosphor autoradiography and gamma counting. Sections with different radioligand binding were exposed to a storage phosphor screen for 72 hours and subsequently scraped off for gamma counting. The densities of the sections ranged from 8 to 90 cpm / mm^2, as measured by gamma counting (efficiency of 60%). Values are expressed as cpm / section. SPS: storage phosphor screen.*

Distribution of [^{125}I]iodophenpropit binding sites in rat brain.

As reported previously, [^{125}I]iodophenpropit binding sites were heterogeneously distributed in rat brain (Figure 1, Figure 3). The densities of [^{125}I]iodophenpropit binding in different brain areas as quantified using storage phosphor method are given in Table 1. [^{125}I]Iodophenpropit binding in the presence of 1 μM (R)α-methylhistamine (Figure 1D) and of 0.3 μM thioperamide (Figure 1C) were homogeneously distributed. Specific binding as defined using (R)α-methylhistamine (1 μM) ranged from 45% to 55% in most areas (cortical, striatal, see Table 2, see page 101) but was small in areas with the lowest H_3-receptor densities (cerebellum: 10 ± 8%, n=8; brain stem: 24 ± 14%, n=8). The highest densities of [^{125}I]iodophenpropit binding sites were found in the substantia nigra, the cerebral cortex, the olfactory tubercle, the nucleus accumbens, the caudate putamen and the anterior amygdaloid area (Table 1). Moderate densities were present in the hippocampus, thalamus, hypothalamus, and the caudal part of the amygdala. The cerebellum and the brain stem showed the lowest [^{125}I]iodophenpropit binding densities. In some brain areas a heterogeneous distribution pattern was observed. A laminar distribution was seen in the cortex with high densities in the upper frontal layers and lower temporal layers. In the hippocampus a relatively dense labeling was found in dentate gyrus.

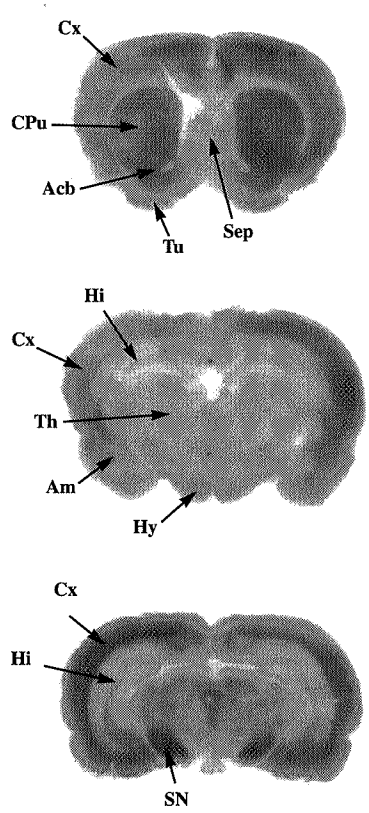

Figure 3. *Autoradiograms of [^{125}I]iodophenpropit binding to transversal sections of rat brain. Abbreviations used: Acb, nucleus accumbens; CPu, caudate putamen; Cx, cortex; Hi, hippocampus; Hy, hypothalamus; Sep, septum; SN, substantia nigra; Th, thalamus; Tu, olfactory tubercle.*

Competition binding curves of histamine H_3-receptor ligands

In order to study the possible heterogeneity of [^{125}I]iodophenpropit binding sites in rat brain competition binding experiments were performed in ten different brain areas using a chemically heterogeneous group of H_3-antagonists (Figure 4). In all areas examined the H_3-antagonists showed monophasic competition binding curves (i.e. curves are best fitted to a one site model, $P > 0.05$) with a high affinity for the [^{125}I]iodophenpropit binding sites (Table 2, Figure 5). Statistical analysis revealed no significant differences in K_D - values of the antagonists for the [^{125}I]iodophenpropit binding sites between the different

***Table 1.** Comparison of [^{125}I]iodophenpropit and [^{3}H](R)α-methylhistamine binding sites in rat brain.*

	radioligand binding (% of total cerebral cortex)	
	[^{125}I]iodophenpropit[1)] *(autoradiography)*	[^{3}H](R)α-methylhistamine[2)] *(membrane preparations)*
anterior cerebral cortex	97 ± 8	108 ± 2
medial cerebral cortex	96 ± 11	100 ± 2
posterior cerebral cortex	110 ± 13	85 ± 6
olfactory tubercle	121 ± 13	103 ± 8
hippocampus	53 ± 8	48 ± 4
caudate putamen	127 ± 10	108 ± 11
nucleus accumbens	133 ± 7	126 ± 12
septum	77 ± 4	N.D.
hypothalamus (anterior)	67 ± 5	70 ± 2
hypothalamus (posterior)	61 ± 7	54 ± 8
hypothalamus (lateral)	68 ± 4	N.D.
hypothalamus (VMH)	78 ± 13	N.D.
thalamus	54 ± 16	N.D.
anterior amygdaloid area	98 ± 6	N.D.
amygdala (posterior)*	69 ± 10	N.D.
substantia nigra	141 ± 21	97 ± 7
pons	33 ± 14	28 ± 5
cerebellum	8 ± 5	7 ± 3

[1)]Specific binding was determined using 1 μM (R)α-methylhistamine. [2)]Values reported by Pollard *et al.*, 1993. *Including: amygdalohippocampal area (AHi) and posteromedial cortical amygdaloid nucleus (PMCo). N.D.: not described. The density of [^{125}I]-iodophenpropit binding sites in the cortex is 268 fmol/mg of protein (Jansen et al., 1994). Randomized brain sections of three to five rats were used. Values are expressed as mean ± SD of three to five separate determinations of which each was performed at least in triplicate.

brain areas (Table 2). The rank order of potency of the antagonists, based on the average affinities in the ten brain areas is: iodophenpropit (1.9 ± 0.6 nM), iodoproxyfan (2.9 ± 0.8 nM) > thioperamide (4.3 ± 1.0 nM) > VUF4616 (22 ± 6 nM) > burimamide (262 ± 60 nM).

Competition binding curves of the H_3-receptor agonists (R)α-methylhistamine and (S)α-methylhistamine were best fit to a one site model. For both agonists the affinities did not vary between different brain areas (average affinities: 3.3 ± 1.0 nM and 159 ± 115 nM, respectively) and were close to their K_D-values for the high affinity site previously described in membrane preparations of the cerebral cortex (3.5 ± 1.2 nM and 230 ± 97

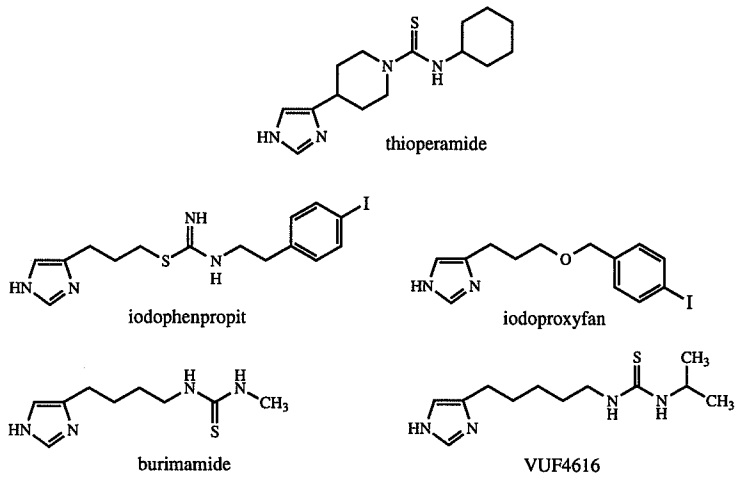

Figure 4. *Structures of thioperamide, iodophenpropit, iodoproxyfan, burimamide and VUF4616.*

Table 2. *Dissociation constants of different H_3-receptor ligands for [^{125}I]-iodophenpropit binding sites in various rat brain areas.*

brain areas	K_D-values, nM						
	thioperamide	iodophenpropit	iodoproxyfan	burimamide	VUF4616	(R)α-MeHa	(S)α-MeHa
frontal cortex	3.2 ± 1.5	1.7 ± 0.4	3.6 ± 2.7	176 ± 64	15 ± 6	4.3 ± 2.6	293 ± 230
parietal cortex	5.1 ± 2.9	1.4 ± 0.5	2.0 ± 1.5	N.D.	24 ± 10	1.9 ± 0.3	241 ± 151
occipital cortex	5.3 ± 4.9	1.9 ± 0.6	1.7 ± 0.9	N.D.	21 ± 3	2.0 ± 0.9	204 ± 163
caudate putamen (D)	2.7 ± 0.6	1.4 ± 0.2	3.6 ± 2.1	289 ± 137	19 ± 5	4.3 ± 1.9	123 ± 58
caudate putamen (V)	3.5 ± 1.0	1.4 ± 0.2	3.6 ± 3.0	273 ± 122	25 ± 12	3.9 ± 2.6	132 ± 72
nucleus accumbens	3.8 ± 2.3	1.3 ± 0.2	2.7 ± 2.2	312 ± 218	17 ± 7	4.5 ± 2.9	138 ± 85
hypothalamus	5.6 ± 1.0	2.4 ± 1.8	2.9 ± 2.9	N.D.	19 ± 2	4.4 ± 1.4	429 ± 343
hippocampus	3.8 ± 4.7	2.5 ± 1.4	3.0 ± 2.7	N.D.	24 ± 6	3.4 ± 2.9	415 ± 205
thalamus	5.1 ± 3.5	3.2 ± 1.2	3.8 ± 3.6	N.D.	21 ± 9	2.2 ± 1.7	365 ± 375
substantia nigra	4.7 ± 2.0	1.5 ± 0.5	2.0 ± 0.9	N.D.	37 ± 8	2.3 ± 1.5	252 ± 194

Transversal sections were incubated with 0.3 nM [^{125}I]-iodophenpropit as described in 'materials and methods'. K_D-Values are expressed as average of three to six separate observations determined in two or three separate experiments. $^*P < 0.05$, as compared to other brain areas using a Student's t-test with a Bonferroni correction. The percentage of specific binding as defined with 1 μM (R)α-methylhistamine was: frontal cortex: 51 ± 7%; parietal cortex: 47 ± 11%; occipital cortex: 49 ± 10%, caudate putamen (V; ventral aspect) 51 ± 6%; caudate putamen (D; dorsal aspect): 48 ± 6%; nucleus accumbens: 53 ± 7%; hippocampus 33 ± 17%; hypothalamus: 30 ± 14%; thalamus: 22 ± 10%; substantia nigra: 55 ± 11%. Abbreviations used: (R)α-MeHa: (R)α-methylhistamine; (S)α-MeHa: (S)α-methylhistamine. Sections were used from the following brain areas: striatal areas and frontal cortex: AP, -1.7 mm to -0.7 mm; hypothalamus, thalamus, hippocampus and parietal cortex: AP, 2.3 mm to 2.8 mm; substantia nigra and occipital cortex: AP, 4.8 mm to 5.8 mm, according to the atlas of Paxinos and Watson (1982).

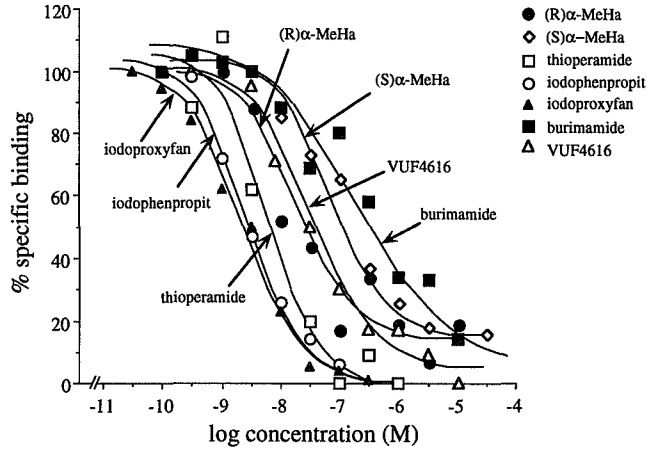

Figure 5. *Competition binding curves of different H_3-receptor ligands in the ventral part of the caudate putamen. Transversal tissue sections were incubated with for one hour [^{125}I]iodophenpropit (0.3 nM) in presence of different concentrations of drugs. Specific binding was determined using thioperamide (0.3 μM).*

nM, respectively, Jansen *et al.*, 1994). The clear difference in the affinity between both enantiomers is in accordance with displacement of [^{125}I]iodophenpropit from histamine H_3-receptor sites. Competition binding curves of the H_3-receptor ligands in the caudate putamen are shown in Figure 5.

Comparison of H_3-agonist and H_3-antagonist displacement

In studies using the H_3-receptor antagonist [^{125}I]iodoproxyfan, it was observed that [^{125}I]iodoproxyfan binding to membranes of rat striatum was only partially displaced by agonists (60% of the total binding), whereas the binding was completely and monophasically displaced by most antagonists, including iodoproxyfan itself (Ligneau *et al.*, 1994). Thus, it seems that about 40% of the binding displaced by H_3-receptor antagonists is not likely to reflect H_3-receptors due to binding of [^{125}I]iodoproxyfan and of the other antagonists with high affinity to a non H_3-receptor site. Recently, our laboratory reported similar findings with [^{3}H]thioperamide (Alves-Rodrigues *et al.*, 1996). Therefore, we have made a comparison between [^{125}I]iodophenpropit binding displaceable by the H_3-agonist (R)α-methylhistamine and by the H_3-antagonist thioperamide. Competition binding curves for (R)α-methylhistamine and for thioperamide reached a plateau at 1 μM and 0.3 μM respectively. In most of the brain areas a small difference was observed between [^{125}I]iodophenpropit binding displaced by (R)α-

methylhistamine (1 μM) and binding displaced by thioperamide (0.3 μM) i.e. the binding displaced by (R)α-methylhistamine as percentage of binding displaced by thioperamide ranging from 80 to 90 percent. More marked differences were observed in the hippocampus (70 ± 24%, P > 0.05), the hypothalamus (72 ± 34%, P > 0.05) and the thalamus (59 ± 27%, n=8, P < 0.05). A significant difference was also found in the frontal cortex and in the occipital cortex (81 ± 16%, n=9, P < 0.05 and 78 ± 18%, n=11, P < 0.05, respectively). Hence, in most of the areas studied a minor non-H_3-receptor component may be included in the competition binding curves for thioperamide which is not recognized by the curve fitting procedure as a separate site, possibly due to its relatively low abundance and / or its small difference in affinity compared to the H_3-receptor. Though, a good correlation was found between the [^{125}I]iodophenpropit binding displaced by (R)α-methylhistamine (1 μM) and by thioperamide (0.3 μM) in the different brain areas (Figure 6). The other H_3-receptor antagonists displaced [^{125}I]iodophenpropit binding approximately to the same level as thioperamide (0.3 μM). For all antagonists studied, the [^{125}I]iodophenpropit binding at this level was not further displaced by addition of (R)α-methylhistamine (1 μM) indicating that these ligands displace essentially the same component as (R)α-methylhistamine (1 μM).

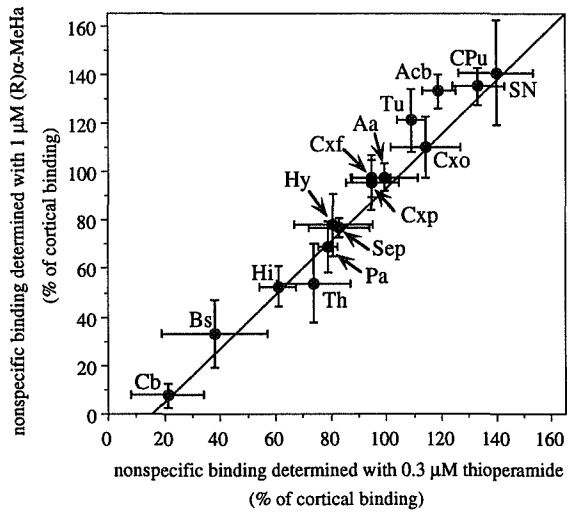

Figure 6. *[^{125}I]Iodophenpropit binding in rat brain areas; comparison between the nonspecific binding defined with (R)α-methylhistamine (1 μM) and with thioperamide (0.3 μM). [^{125}I]Iodophenpropit binding in the different brain areas is expressed as percentage of the cortical binding. Abbreviations: Aa, anterior amygdala; Acb, nucleus accumbens; Bs, brain stem; Cb, cerebellum; CPu, caudate putamen; Cxf, frontal cortex; Cxo, occipital cortex; Cxp, parietal cortex; Hi, hippocampus; Hy, hypothalamus; Pa; posterior amygdala; Sep, septum; SN, substantia nigra; Th, thalamus; Tu, olfactory tubercle.*

Discussion

We have previously introduced [^{125}I]iodophenpropit as the first radiolabelled histamine H_3-receptor antagonist (Jansen *et al.,* 1992). [^{125}I]Iodophenpropit meets the criteria for a suitable radioligand for histamine H_3-receptor binding studies in rat cerebral cortex, showing a high selectivity, reversibility, a high affinity ($K_D = 0.6 \pm 0.2$ nM), saturability ($B_{max} = 268 \pm 119$ fmol/mg) and an acceptable amount of specific binding (50 to 60% of total binding, Jansen *et al.,* 1992; Jansen *et al.,* 1994). Moreover, the binding affinities of different H_3-receptor agonists and antagonists for [^{125}I]iodophenpropit binding sites in the cerebral cortex are in agreement with their potencies measured by functional *in vitro* H_3-receptor test systems, indicating [^{125}I]iodophenpropit binding sites are related to functional histamine H_3-receptor sites.

In the present study we extended the characterization of [^{125}I]iodophenpropit binding in the rat CNS studying different brain areas using quantitative autoradiography. A benefit of the use autoradiography as compared to binding experiments on membrane suspensions is that binding studies performed on tissue sections have a higher anatomical accuracy. In addition, it allows the simultaneous determination of K_D-values in distinct brain areas located on the same section. Radiolabelled tissue sections were quantified using the storage phosphor imaging technique which has on its turn several advantages over conventionally used computer assisted methods i.e.: a wide dynamic range, high accuracy of determination and sensitivity (Kanekal *et al.,* 1995). The method can be used for many β- and γ- emitting radionuclides. Its application for the use of [^{125}I]-labelled ligands has recently been demonstrated for the quantification of autoradiographic images of cholecystokinin receptors in rat brain (Tang *et al.,* 1995). The wide dynamic range of this method was apparent by the high linearity between phosphor image quantification and measurement of radioactivity by gamma counting. Although the image resolution is lower as compared to conventional film, storage phosphor autoradiography proves to be a very useful method for image quantification.

A detailed mapping of [^{3}H](R)α-methylhistamine binding sites has been described by Pollard et al. (1993). The quantitative distribution of [^{125}I]iodophenpropit binding sites as reported in the present study is comparable to the distribution of [^{3}H](R)α-methylhistamine binding sites. However, especially in the substantia nigra, densities found with [^{125}I]iodophenpropit were higher as compared to [^{3}H](R)α-methylhistamine binding sites. Although a straightforward explanation for this difference cannot be given, these findings may be related to the role of G-protein interaction involved in binding of the radiolabelled agonist. The distribution of [^{125}I]iodophenpropit binding sites is also in general agreement with the qualitative distribution of the binding sites as described with

[^{3}H]S-methylthioperamide (Yanai *et al.*, 1994) and [^{125}I]iodoproxyfan (Ligneau *et al.*, 1994).

As mentioned previously (Arrang *et al.*, 1987; Pollard *et al.*, 1993), there is no consistent overlap of the H_3-receptor densities with the distribution of the histaminergic cell bodies and varicose fibers in the CNS. It is tempting to relate this phenomenon to the presence of the H_3-receptor as a heteroreceptor. A modulatory effect of H_3-receptor activation on the release of various transmitters, such as serotonin (Fink *et al.*, 1990), noradrenaline (Schlicker *et al.*, 1989), acetylcholine (Clapham & Kilpatrick, 1992) and neuropeptides (Matsubara *et al.*, 1992) has been reported. Moreover, postsynaptic localization of histamine H_3-receptors have been indicated (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). Finally, the 'mismatch' between the histaminergic system and histamine H_3-receptors could, in a speculative way, also be explained from a different expression of histamine H_3-receptors in different brain areas. However, at present there is no evidence for the latter suggestion.

Some evidence exists that the actions through H_3-receptors are mediated by a heterogeneous population of receptor sites. In receptor binding studies the antagonists burimamide and thioperamide biphasically displaced the radiolabelled H_3-receptor agonist [^{3}H]N$^{\alpha}$-methylhistamine from rat brain (West *et al.*, 1990b; Cumming & Gjedde, 1994). From these observations the existence of H_{3A} and H_{3B}-receptor subtypes has been suggested (West *et al.*, 1990b). The biphasic nature of burimamide displacement curves has been confirmed by other laboratories, using the agonists [^{3}H](R)α-methylhistamine (Arrang *et al.*, 1990), [^{3}H]N$^{\alpha}$-methylhistamine (Kathmann *et al.*, 1993; Brown *et al.*, 1996) and the antagonist [^{125}I]iodophenpropit (Jansen *et al.*, 1994). However, this biphasic displacement was not observed in some other reports (West *et al.*, 1990a; Kilpatrick & Michel, 1991; Clark & Hill, 1995). To our knowledge, for thioperamide the biphasic nature was described in two additional studies (Clark & Hill, 1995; Brown *et al.*, 1996).

At present, data with respect to the regional distribution of the possible subtypes are scarce. In the rat striatum and the surrounding cortical areas the ratios between the two putative binding sites for burimamide showed no variation (Cumming & Gjedde, 1994). No data are available in other brain areas with this respect. Yet, information about binding affinities of H_3-receptor ligands in rat brain is largely confined to the cerebral cortex and the striatum. Therefore, we have determined the affinities for the [^{125}I]iodophenpropit binding sites of a chemically heterogeneous group of H_3-antagonists in ten different rat brain areas. From these studies no heterogeneity of the [^{125}I]iodophenpropit binding sites was found in the different brain areas studied. For all antagonists, displacement curves were best fitted according to a one site model showing no significant variation in K_D-values between the different brain areas. The K_D-values of iodophenpropit, thioperamide

Table 3. *Comparison of the potencies of the histamine H_3-receptor agonists and antagonists, as determined in receptor binding experiments and in functional studies.*

	RECEPTOR BINDING AFFINITIES					FUNCTIONAL POTENCIES	
	cerebral cortex (membrane suspensions)[1]				cerebral cortex (autoradiography)[2]	cerebral cortex[3]	jejunum[4]
	[^{3}H]NAMH binding (pK_D)		[^{125}I]Ipp binding (pK_D)[b]		[^{125}I]Ipp binding (pK_D)	(pA_2)	(pA_2)
H_3-antagonists:							
iodophenpropit	N.D.		9.0		8.8	9.4[a]	9.6[b]
iodoproxyfan	9.5[c]		8.6[a]		8.6	9.0[c, *]	9.0[c, *]
thioperamide	8.7[d]		8.4		8.3	8.7[e]	8.9[f]
	($pK_{D,1}$)	($pK_{D,2}$)	($pK_{D,1}$)	($pK_{D,2}$)			
burimamide	7.0[g]	5.5[g]	7.7	6.1	6.8	6.8[e]	7.0[f]
VUF4616	N.D.	N.D.	9.4[a]	7.6[a]	7.7	N.D.	7.7[f]
H_3-agonists:						(pD_2)	(pD_2)
			(pK_H)	(pK_L)			
(R)α-methylhistamine	9.3[d]		8.5	5.9	8.6	7.9[h]	7.8[f]
(S)α-methylhistamine	7.3[i, **]		6.6	5.0	6.6	6.4[h]	6.4[f]

[1]As determined on rat brain; [2]Average values of the frontal, parietal and occipital cortex; [3]H_3-Receptor mediated inhibition of [^{3}H]noradrenaline release (except for iodophenpropit: [^{3}H]serotonin release); [4]H_3-Receptor mediated inhibition of the neurogenic contraction of the guinea-pig jejunum. *Iodoproxyfan also acts as a partial agonist on these assays (Schlicker *et al.*, 1996); **[^{3}H](R)α-methylhistamine binding. [a]Unpublished observation; [b]Jansen *et al.*, 1994; [c]Schlicker *et al.*, 1996; [d]Brown *et al.*, 1996; [e]Schlicker *et al.*, 1992; [f]Vollinga *et al.*, 1995; [g]West *et al.*, 1990b; [h]Leurs *et al.*, 1996; [i]Arrang *et al.*, 1990. Abbreviations: [^{3}H]NAMH, [^{3}H]N$^\alpha$-methylhistamine; [^{125}I]Ipp, [^{125}I]-iodophenpropit.

and iodoproxyfan found in this study are in accordance with the values obtained from rat cerebral cortex membrane preparations (Table 3). For the histamine H_3-receptor antagonist burimamide and its 5-fold more potent derivative VUF4616 (Vollinga *et al.*, 1995) the monophasic nature of the competition binding curves was rather unexpected as we have previously observed two distinct affinities of burimamide (Jansen *et al.*, 1994) and of VUF4616 (unpublished observations) for [^{125}I]iodophenpropit binding sites in whole cerebral cortex membrane suspensions. The K_D-values observed in the autoradiographic study are in agreement however with their antagonistic activities (Table 3). A possible explanation of the differences in our studies, and of those reported in literature, with respect to discrimination between the two binding sites may relate to the relative small difference in binding affinity (about 40-fold) of the ligands for the two different sites. The significant 'increased goodness' of the two-site fit (as compared to the one-site fit) will decrease when the difference between the affinities of two different sites becomes smaller. Hence, the failure to detect different binding sites may be related to this phenomenon. Additionally, the relatively higher variance in the autoradiographic studies (as compared to membrane preparation experiments) may also attribute to the failure of finding two sites. Nevertheless, if histamine H_3-receptor subtypes would exist, they would probably show a distinct distribution in the different brain areas studied, which

would likely be reflected in the displacement curves, as the difference between the two affinities is large enough to distinguish the subtypes individually.

An important criterion for the establishment of receptor subtypes is that they are related to distinct functional receptor responses. Presently, the existence of H_{3A}- and H_{3B}-receptors as suggested from receptor binding experiments has not much support from functional studies. West et al. (West *et al.*, 1990b) suggested these subtypes to be related to modulation of histamine release and histamine synthesis, respectively. The histamine H_3-receptors inhibiting the release of noradrenaline in mouse brain cortex slices have been suggested to represent the H_{3A}-receptor subtype (Schlicker *et al.*, 1992; Schlicker *et al.*, 1994a). To our knowledge, additional functional responses in brain tissue related to the H_{3B}-receptor have never been observed however. It should be noted that a possible negative co-operativity could also explain the biphasic nature of burimamide displacement curves. Moreover, the biphasic nature of the displacement curves of thioperamide has been proposed to arise from different conformational states of the histamine H_3-receptor, dependent on the buffer composition (Clark & Hill, 1995). In conclusion, the possible existence of H_3-receptor heterogeneity in the CNS needs further exploration.

Similarly to the observations for H_3-receptor antagonists, the H_3-receptor agonists (R)α-methylhistamine and (S)α-methylhistamine showed no variation in affinities between different brain areas. Curves for (R)α-methylhistamine and (S)α-methylhistamine were best fitted to a one-site model. The affinities are close to the values of the high affinity binding sites found in membrane preparations of cerebral cortex (see Table 3). Hence, it may be speculated that in the autoradiographic preparation the low affinity site of the agonist is not recognized as a separate site as a consequence of a higher fraction in the high affinity state as compared to membrane preparations. In all ten brain areas a clear difference in affinity between the stereoisomers (R)α-methylhistamine and (S)α-methylhistamine was found, which is in accordance with the displacement of [^{125}I]iodophenpropit from H_3-receptor sites by these agonists.

In studies using [^{125}I]iodoproxyfan, it was observed that [^{125}I]iodoproxyfan binding to membranes of rat striatum was partially displaced by agonists (60% of the total binding), whereas the binding was completely and monophasically displaced by most antagonists, including iodoproxyfan itself (Ligneau *et al.*, 1994). Apparently, [^{125}I]iodoproxyfan binds with a comparable high affinity to a non-H_3-receptor site, representing about 40% of the total [^{125}I]iodoproxyfan. For the binding of [^{3}H]thioperamide to rat cerebral cortical membranes, our group recently obtained similar findings (Alves-Rodrigues *et al.*, 1996). Based on these findings, in the present study we have made a similar comparison with [^{125}I]iodophenpropit. In most of the brain areas a difference of 10 to 20% was found between [^{125}I]iodophenpropit binding displaced by the H_3-receptor

agonist (R)α-methylhistamine and by the H_3-receptor antagonist thioperamide. Although, a similar phenomenon is observed with [^{125}I]iodophenpropit, the non-H_3-receptor component included in antagonist displacement of [^{125}I]iodophenpropit seems to be smaller as compared to [^{125}I]iodoproxyfan i.e. 15% versus 40%, in striatal tissue. Yet, in other regions the non-H_3-receptor component of [^{125}I]iodophenpropit could amount 30-40% (e.g. hippocampus and thalamus). Using rat hippocampal membrane preparations we recently obtained similar results (Alves-Rodrigues, unpublished observations). With respect to the nonspecific binding of radiolabelled H_3-antagonists it is noteworthy that a new tritiated derivative of thioperamide has been recently reported, i.e. [^{3}H]GR168320 (Brown *et al.*, 1996). The binding of [^{3}H]GR168320 to rat cerebral cortex membranes was equally and almost fully displaced both, by H_3-agonists and by H_3-antagonists. The apparent absence of non-H_3-receptor components in the binding of [^{3}H]GR168320 to rat cerebral cortex membranes might be related to the high affinity of [^{3}H]GR168320 for the cortical H_3-receptors (K_D of 0.1 nM), as compared to [^{3}H]thioperamide (K_D of 0.8 nM, Alves-Rodrigues *et al.*, 1996), resulting in a more selective labelling of histamine H_3-receptors.

Iodophenpropit and thioperamide have recently been screened on a large variety of receptor assays (Leurs *et al.*, 1995a). Iodophenpropit showed a high selectivity towards H_3-receptors. The difference in affinity for other receptors tested was 100-fold or more, except for 5-HT_3-receptors (K_D = 11 nM, 10-fold lower as compared to H_3-receptor). Also thioperamide displayed moderate activity towards 5-HT_3-receptors (K_D = 120 nM, 30-fold lower as compared to H_3-receptor). Considering the affinity of iodophenpropit for 5-HT_3-receptors and the low density of 5-HT_3-receptors in the CNS (< 10 fmol/mg, Kilpatrick *et al.*, 1990), it can be predicted that binding of [^{125}I]iodophenpropit to 5-HT_3-receptors is negligible at the experimental conditions used. Indeed, [^{125}I]iodophenpropit binding to rat cortical membranes was not displaced by the selective 5-HT_3-receptor antagonist ondansetron (Leurs *et al.*, 1995a). For [^{3}H]thioperamide, there is some evidence that its non-H_3-receptor binding includes binding to cytochrome P_{450} isoenzymes (Alves-Rodrigues *et al.*, 1996). Whether the binding of [^{125}I]iodophenpropit to these proteins contributes to the non-H_3-receptor binding has to be investigated.

In conclusion, in the present study we have shown that [^{125}I]iodophenpropit is a suitable radioligand for the characterization of histamine H_3-receptors throughout the rat brain. Storage phosphor autoradiography proves to be a valuable method for the quantification of the radiolabelled autoradiographic images. Specific binding was detected throughout the brain, with densities essentially the same as reported for the agonist [^{3}H](R)α-methylhistamine. The difference observed between binding displaceable by agonists and

antagonists in some areas (thalamus, hippocampus and hypothalamus) has implications for the definition of the specific binding in these regions. From displacement studies of [^{125}I]iodophenpropit with a chemically heterogeneous group of H_3-antagonists we have not been able to provide any further evidence for a heterogeneity of H_3-receptors in the rat CNS.

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- *Chapter 6* -

Unilateral 6-hydroxydopamine denervation of the nigrostriatal pathway in the rat differentially affects histamine H_3-receptors in the striatum and in nuclei of striatal efferents

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Abstract

We have studied the interaction between histamine H_3-receptors and the dopaminergic neuron system in rat brain. Rats were unilaterally denervated with 6-hydroxydopamine injected into the substantia nigra and the ventral tegmental area. The effect of dopaminergic lesions on binding of the histamine H_3-receptor antagonist [^{125}I]iodophenpropit was studied at three and at twelve weeks post-lesioning, using quantitative receptor autoradiography. Radioactive images were quantified applying storage phosphor autoradiography. Denervation caused an increase of [^{125}I]iodophenpropit binding in the dorsal aspect of the ipsilateral striatum both at three weeks (+18%, as compared to the contralateral side) and at twelve weeks (+12%) post lesioning. 6-Hydroxydopamine treatment also affected [^{125}I]iodophenpropit binding in striatal output nuclei. A pronounced increase was observed in the entopeduncular nucleus (+44% to +65%) and in the substantia nigra (+64% to +81%) three and twelve weeks post-lesioning. In the globus pallidus a small decrease (~6%) of [^{125}I]iodophenpropit binding was observed, which was only significant at twelve weeks post-lesioning. In general, similar changes of [^{125}I]iodophenpropit binding were observed at three months as compared to twelve months post-lesioning. Hence, these changes seem to be related directly to the sustained destruction of dopaminergic neurons in 6-hydroxydopamine treated rats. The changed histamine H_3-receptor binding may be of importance in the pathology of motor disorders like Parkinson's disease.

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Introduction

The role of histamine as a neurotransmitter in the CNS has been well established (Schwartz *et al.*, 1991; Onodera *et al.*, 1994; Schwartz *et al.*, 1995). The histaminergic system has been described to play a role in different processes such as arousal state, sleep and wakefulness, locomotor activity, feeding, cognition and pituitary hormone secretion (for review see: Onodera *et al.*, 1994; Schwartz *et al.*, 1995). Histaminergic neurons originate from the posterior hypothalamic area and send varicose fibers towards all parts of the CNS through ascending and descending projections (Tohyama *et al.*, 1991; Panula & Airaksinen, 1991; Wouterlood & Steinbusch, 1991; Schwartz *et al.*, 1995). Release and synthesis of neuronal histamine are both inhibited upon activation of the histamine H_3-receptor, the autoreceptor of histaminergic neurons (Arrang *et al.*, 1983). Autoradiographic studies showed that there is no clear overlap between the distribution of H_3-receptors and the distribution of histaminergic neurons (Arrang *et al.*, 1987; Pollard *et al.*, 1993). This observation may be partly explained by the presence of the H_3-receptor as a heteroreceptor (for review see: Schlicker *et al.*, 1994). Activation of the histamine H_3-receptor has been shown to inhibit the release of noradrenaline, serotonin and acetylcholine from rat brain slices (Schlicker *et al.*, 1994). In addition, from autoradiographic studies, postsynaptic H_3-receptors have been proposed (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). Intrastriatal quinolinic acid or kainic acid lesions, destroying postsynaptic components (Schwarcz *et al.*, 1983; Nicholson *et al.*, 1995), caused a marked reduction of H_3-receptors in the striatum as evidenced from receptor binding studies with the radiolabelled H_3-agonists [^{3}H](R)α-methylhistamine (Pollard *et al.*, 1993; Ryu *et al.*, 1994) and [^{3}H]N$^\alpha$-methylhistamine (Cumming *et al.*, 1991). Lesion of dopaminergic neurons originating from the substantia nigra, pars compacta resulted in an increase of [^{3}H](R)α-methylhistamine binding in the striatum and in the substantia nigra (Ryu *et al.*, 1994; Ryu *et al.*, 1996). Hence, the H_3-receptor may play a functional role in the nigrostriatal tract, which was shown to be endowed with histamine H_3-receptors.

Up till now, autoradiographic reports on histamine H_3-receptors in the nigrostriatal tract have focussed almost exclusively on transversal sections of the rostral striatum and of the substantia nigra. Moreover, in these studies predominantly radiolabelled histamine H_3-receptor agonists were used to determine the density of H_3-receptors. Radiolabelled H_3-receptor agonists show a rather complex receptor binding profile as a consequence of involvement of coupling of the agonist-receptor complex to G-proteins (for review see: Leurs *et al.*, 1995).

In the present study we investigated the interaction between H_3-receptors and the dopaminergic system, using the iodinated H_3-receptor antagonist [^{125}I]-iodophenpropit (Jansen *et al.*, 1992; Jansen *et al.*, 1994). Dopaminergic neurons originating from the substantia nigra and the ventral tegmental area were treated with the neurotoxin 6-hydroxydopamine (6-OHDA) and subsequently, the effect of denervation on [^{125}I]-iodophenpropit binding was studied with quantitative autoradiography.

Materials and methods

Lesions with 6-OHDA

Male rats (T-Maze Bright (TMB), 200-250g, Central Animal Facility, Maastricht University) were housed in pairs under standard conditions (12 hr light-dark cycle, 23°C, 55% relative humidity) in macrolon cages (25x20x30 cm) receiving water and standard food pellets (Hope Farms) *ad libitum.* Rats were subjected to unilateral 6-OHDA lesioning. Two hours prior to lesion-surgery rats received an injection with desmethylimipramine (25mg/kg i.p., dissolved in 0.9% NaCl), to prevent 6-OHDA uptake into noradrenergic neurons. Under anaesthesia, using a combination of xylazine (2mg/kg, s.c.) and ketamine (50mg/kg, i.m.), 6-OHDA was injected stereotaxically in the ventral tegmental area and in the medial forebrain bundle. 6-OHDA was dissolved in saline (0.9% NaCl) containing 0.02% (wt/vol) ascorbic acid. Following the injection (injection rate of 1 µl/min) the needle was left in place for an additional minute. Co-ordinates (according to Paxinos & Watson, 1982) and volumes used were: B -4.0 mm, L 0.8 mm, V 8.0 mm; 2.0 µl and B -4.4 mm, L 1.1 mm, V 7.8 mm; 2.5 µl, for the ventral tegmental area and the medial forebrain bundle, respectively.

Receptor autoradiographic procedure

Male TMB rats were decapitated and the brains were immediately removed and frozen using dry ice. Transversal cryostat sections (14 µm) were cut, mounted onto gelatin/chromalum-coated glass slides and stored at -80°C until use. The tissue sections were thawed and incubated with 0.3 nM [^{125}I]-iodophenpropit for 60 minutes at 37°C in 50 mM Tris-HCl buffer containing 145 mM NaCl, 5 mM $MgCl_2$ and 0.25% BSA (pH 7.4). Nonspecific binding was determined with thioperamide (0.3 µM). In displacement experiments tissue sections were incubated (in duplicate) with [^{125}I]-iodophenpropit (0.3 nM) in the presence of different concentrations of cold iodophenpropit (0.1 nM to 100 nM). The tissue sections were randomized prior to the experiment to minimize the effect of possible rostro-caudal variation in receptor densities. To stop the incubations the sections were rinsed once with ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM $MgCl_2$, 145 mM NaCl; pH 7.4 at 4°C) and were subsequently washed twice for 15 minutes in

ice-cold Tris-HCl buffer and 15 seconds in ice-cold distilled water. Sections were dried by a stream of cold air and were exposed to Hyperfilm (Amersham International, U.K.) for 20 hours and subsequently to a storage phosphor screen. Phosphor screens were scanned with a Phosphor Imager® (Molecular Dynamics, Sunnyvale, CA, USA) to obtain the digitized images. Proper images were obtained already several hours after exposure. To increase the resolution of the digitized images the screens were exposed up to three days. In some experiments, tissue sections were scraped from the slides with a razor blade in order to count the radioactivity by an LKB gamma counter.

Data analysis and statistical evaluation:

The storage phosphor images were analyzed and quantified using the program ImageQuant® (Molecular Dynamics). Brain areas of interest (100 to 600 pixels, pixel size 88 x 88 μm) were selected and the density was expressed as the average counts per pixel-value in the selected brain areas. A linear relation between quantification with the phosphor imaging method compared to direct gamma counting was observed over the range of densities used in the experiments. (For a more detailed description of this method: see Kanekal *et al.*, 1995; Tang *et al.*, 1995). Specific [^{125}I]-iodophenpropit binding was calculated by substracting the nonspecific binding from the total binding. In each experiment specific binding of the ipsilateral side was expressed as percentage of the binding in the contralateral side. The specific binding in the ipsilateral side was compared to the contralateral side (population mean of 100%) by a one sample unpaired Student's t-test using the program StatWorks™1.2 on a Macintosh computer. Differences were considered statistically significant at a probability level of 5%. Competition binding experiments were evaluated using the non-linear curve fitting program LIGAND (Munson & Rodbard, 1980) on a Macintosh computer as previously described (Jansen *et al.*, 1994). Binding curves were fit (unweighed) to a one and a two site model respectively, and statistically tested on the increasing goodness of the fit for a model with additional parameters using a probability level of 5%. Displacement curves of iodophenpropit were best fitted to a one-site model. Dissociation constants of iodophenpropit in lesioned and unlesioned side of the sections were compared using unpaired two-tailed Student's t-tests.

Ligands

[^{125}I]-Iodophenpropit (specific activity of 1900 Ci mmol$^{-1)}$ was synthesized at the Radionuclide Center, Leiden/Amsterdam Center for Drug Research (Vrije Universiteit, Amsterdam), as described previously (Menge *et al.*, 1992). The following drugs were used: iodophenpropit dihydrobromide (laboratory stock), thioperamide maleate (laboratory stock) All compounds (solutions of 1-10 mM) were dissolved in distilled water or in Tris-HCl buffer and were further diluted in Tris-HCl buffer.

Results

In a separate study, rats received identical 6-OHDA lesions and the brains were perfusion-fixated with either glutaraldehyde or paraformaldehyde for dopamine or tyrosine-hydroxylase-immunohistochemistry, respectively. (Details about both procedures have been published previously by Steinbusch *et al.*, 1988 and Steinbusch *et al.*, 1991). 6-OHDA lesions of dopaminergic neurons originating from the substantia nigra pars compacta and the ventral tegmental area resulted in selective and prolonged loss of dopaminergic neuronal pathways in the CNS (Steinbusch *et al.*, 1988; Steinbusch *et al.*, 1991).

Lesions with 6-OHDA resulted in a moderate increase of [^{125}I]iodophenpropit binding in the dorsal aspects of the striatum (Table 1). A significant increase was found in the dorsolateral aspect (+18%) at three weeks post-lesioning and in the dorsomedial aspect (+12%) at twelve weeks post-lesioning. [^{125}I]Iodophenpropit binding was markedly and significantly increased in the entopeduncular nucleus (+44%) and in the substantia nigra pars reticulata (+81%), at three weeks post-lesioning (Table 1). A similar increase of

Table 1. *Effect of lesions with 6-OHDA on [^{125}I]-iodophenpropit binding sites in rat brain.*

	[^{125}I]-iodophenpropit binding (% of contralateral site)	
	time after lesion	
brain area	3 weeks	12 weeks
cerebral cortex	101 ± 4	100 ± 11
striatum:		
DM	114 ± 9	112 ± 6*
DL	118 ± 6*	113 ± 9
VM	108 ± 6	104 ± 7
VL	110 ± 6	104 ± 10
globus pallidus	95 ± 7	94 ± 3*
entopeduncular nucleus	144 ± 14*	165 ± 7*
substantia nigra	181 ± 12*	164 ± 18*

*P < 0.05, as compared to the contralateral side. Tissue sections were incubated with 0.3 nM [^{125}I]-iodophenpropit as described in 'materials and methods'. Values are expressed as average ± SD of at least three separate experiments with sections obtained from three or four 6-OHDA lesioned rats (3 and 12 weeks post-lesion interval, respectively). Abbreviations: DM, dorsomedial. DL, dorsolateral; VM, ventromedial; VL, ventrolateral.

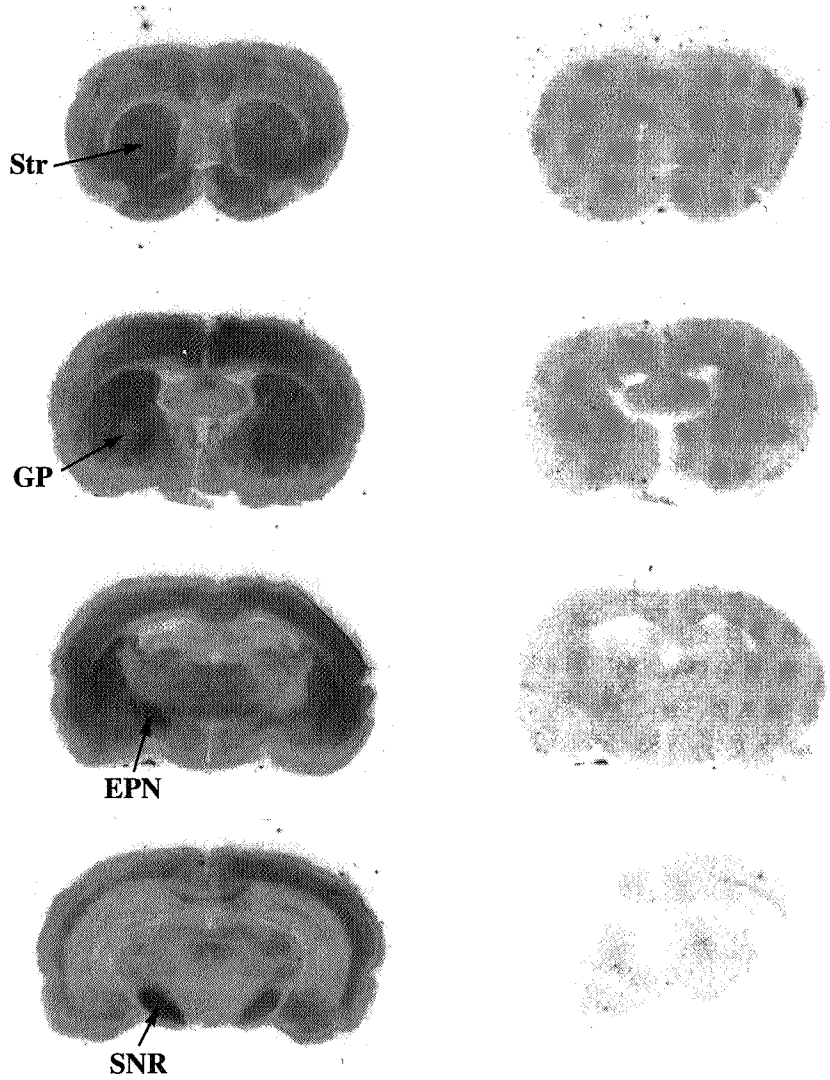


Figure 1. *Autoradiograms of [^{125}I]-iodophenpropit binding after lesions with 6-OHDA. Shown are transversal sections of rats at twelve weeks after 6-OHDA lesions. Sections were incubated with 0.3 nM [^{125}I]-iodophenpropit as described in 'materials and methods'. Left column: total binding; right column: binding in the presence of 0.3 μM thioperamide. The left side of each section corresponds to the 6-OHDA lesioned side. Abbreviations: Str, striatum; GP, globus pallidus; EPN: entopeduncular nucleus; SNR, substantia nigra.*

[^{125}I]iodophenpropit binding in both areas was also observed at twelve weeks post lesioning (Table 1, Figure 1). Analysis of the autoradiograms revealed a marginal decrease of [^{125}I]iodophenpropit binding in the globus pallidus, both at three and twelve weeks post lesioning (Figure 1). A significant reduction (-6%) of [^{125}I]iodophenpropit binding in the globus pallidus was found at twelve weeks after the lesions (Table 1). No interhemispheric differences of [^{125}I]iodophenpropit binding were observed in cortical areas (Table 1). 6-OHDA lesions caused no differences in K_D-values of [^{125}I]iodophenpropit binding between ipsilateral and contralateral hemispheres of the cortex, striatum and the substantia nigra (Table 2, Figure 2).

Table 2. *Dissociation constants of iodophenpropit in 6-OHDA lesioned rats.*

brain area	K_D-value (nM)	
	ipsilateral side	*contralateral side*
cortex	1.7 ± 0.3	1.7 ± 0.3
striatum, dorsal aspect	1.9 ± 0.9	1.3 ± 0.3
striatum, ventral aspect	1.5 ± 0.2	1.3 ± 0.3
globus pallidus	ND.	ND.
entopeduncular nucleus	ND.	ND.
substantia nigra	1.4 ± 0.4	1.4 ± 0.6

Dissociation constants were determined from displacement of 0.3 nM [^{125}I]-iodophenpropit with cold iodophenpropit (see 'materials and methods' section). K_D-Values are expressed as average ± SD of three to five separate experiments. ND, not determined.

Discussion

The histamine H_3-receptor was identified as the autoreceptor of histaminergic neurons in the brain (Arrang *et al.*, 1983). Soon it became clear that the histamine H_3-receptor is not solely present as an autoreceptor. Conclusive evidence was provided for the occurrence of histamine H_3-heteroreceptors modulating the release of various neurotransmitters (for recent review: see (Schlicker *et al.*, 1994). Moreover, from receptor autoradiographic studies the existence of postsynaptic H_3-receptors has been indicated (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). Hence, the H_3-receptor may play an important role both, in regulating the activity of histaminergic neurons and in the cross-talk of neuronal and non-neuronal histamine with other neuronal systems.

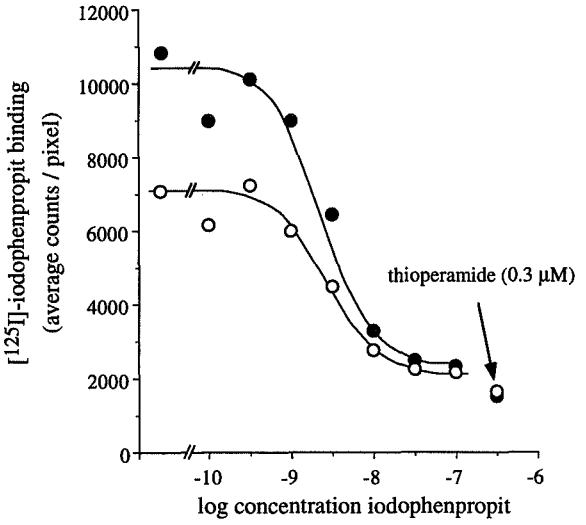


Figure 2. *Competition binding curves for iodophenpropit in the substantia nigra. Total [^{125}I]-iodophenpropit binding is expressed as average counts per pixel in the substantia nigra pars reticulata. Curves are from a single experiment with duplicate determinations. Symbols: (○) control side; (●) lesioned side. Indicated with the arrow: total [^{125}I]-iodophenpropit binding displaced by 0.3 µM thioperamide.*

We have studied the interaction between histamine H_3-receptors and the dopaminergic system in rat brain. The effect of unilateral lesions of dopaminergic neurons induced by the neurotoxin 6-OHDA on the binding of the histamine H_3-receptor antagonist [^{125}I]iodophenpropit to rat fore- and midbrain was examined. [^{125}I]Iodophenpropit was previously demonstrated to be a suitable radioligand to study characteristics and distribution of histamine H_3-receptors in rat brain (Jansen *et al.*, 1992; Jansen *et al.*, 1994). In brief, unilateral 6-OHDA denervation of the nigrostriatal dopaminergic pathway in rat brain moderately increased [^{125}I]iodophenpropit binding in the dorsal aspects of the striatum. Additionally, lesions also altered [^{125}I]iodophenpropit binding in striatal output nuclei. A pronounced increase of [^{125}I]iodophenpropit binding was found in the substantia nigra (SNR) and in the entopeduncular nucleus (EPN). In the globus pallidus (GP) a small but significant decrease of [^{125}I]iodophenpropit binding was observed at twelve weeks after 6-OHDA lesions. In general, comparable changes of [^{125}I]iodophenpropit binding were found at three and at twelve months post-lesioning. The static nature of the changed [^{125}I]iodophenpropit binding may directly be related to the sustained denervation of dopaminergic neurons in the brains of 6-OHDA treated rats (Steinbusch *et al.*, 1988). Lesions with 6-OHDA did not change the dissociation

constants of iodophenpropit (in the cortex, striatum and the SNR). Consequently, changes of [^{125}I]iodophenpropit binding are likely to reflect changes in densities of [^{125}I]iodophenpropit binding sites.

A previous report described the effect of 6-OHDA lesions in rat brain on histamine H_3-receptor binding using the histamine H_3-receptor agonist [^{3}H](R)α-methylhistamine as a radioligand (Ryu *et al.*, 1994). The increased binding of [^{3}H](R)α-methylhistamine in the dorsal striatum (+20%) and in the SNR (+70%) as observed by Ryu *et al.* are comparable to the increased binding of the radiolabelled antagonist [^{125}I]iodophenpropit in these areas. In addition, in our study, we observed changes of [^{125}I]iodophenpropit binding in two other areas i.e. an increase in the EPN and a decrease in the GP. In another autoradiographic study using the agonist [^{3}H]Nα-methylhistamine, dopaminergic lesions were reported not to affect radioligand binding (Cumming *et al.*, 1991) to rat fore- and midbrain. In the latter study, rats were sacrificed already seven days post-lesioning. Possibly, the increased radioligand binding was not yet apparent at this relatively short time interval. It has been demonstrated that changes of dopamine receptor binding were only found in animals with >90% lesion of the nigrostriatal pathway, thus showing the requirement of a fully completed lesion for the altered receptor densities to be detected (Pan *et al.*, 1985). In a recent study by Ryu et al., a marked increase of radiolabelled agonist binding was already observed at one week after lesions with 6-OHDA however (Ryu *et al.*, 1996).

The increased binding of [^{125}I]iodophenpropit in the dorsal striatum and the unaffected [^{125}I]iodophenpropit binding in the ventral striatum of 6-OHDA denervated rats suggests that striatal histamine H_3-receptors are not predominantly located presynaptically on dopaminergic afferents. In accordance with this suggestion, H_3-receptor mediated modulation of dopamine release from rat striatal slices could not be demonstrated so far (Smits & Mulder, 1991; Schlicker *et al.*, 1993).

Histamine H_3-receptors in the striatum may be tonically regulated by neuronal dopamine released from dopaminergic neurons. Additionally, striatal H_3-receptors may also be tonically influenced by neuronal histamine. Electrocoagulation of the lateral hypothalamic area, destructing both histaminergic afferentiation and other aminergic ascending pathways, resulted in a marked increase of [^{3}H](R)α-methylhistamine binding in the striatum (Pollard *et al.*, 1993). Moreover, inhibition of histamine synthesis with the histidine decarboxylase inhibitor α-fluoromethylhistamine increased the binding of the H_3-receptor agonist [^{3}H](R)α-methylhistamine in the striatum of developing rats (Ryu *et al.*, 1995). The rat striatum has been demonstrated to contain a diffuse network of histaminergic varicose fibers (Steinbusch *et al.*, 1986). Thus, striatal histamine H_3-receptors may be tonically regulated by neuronal histamine.

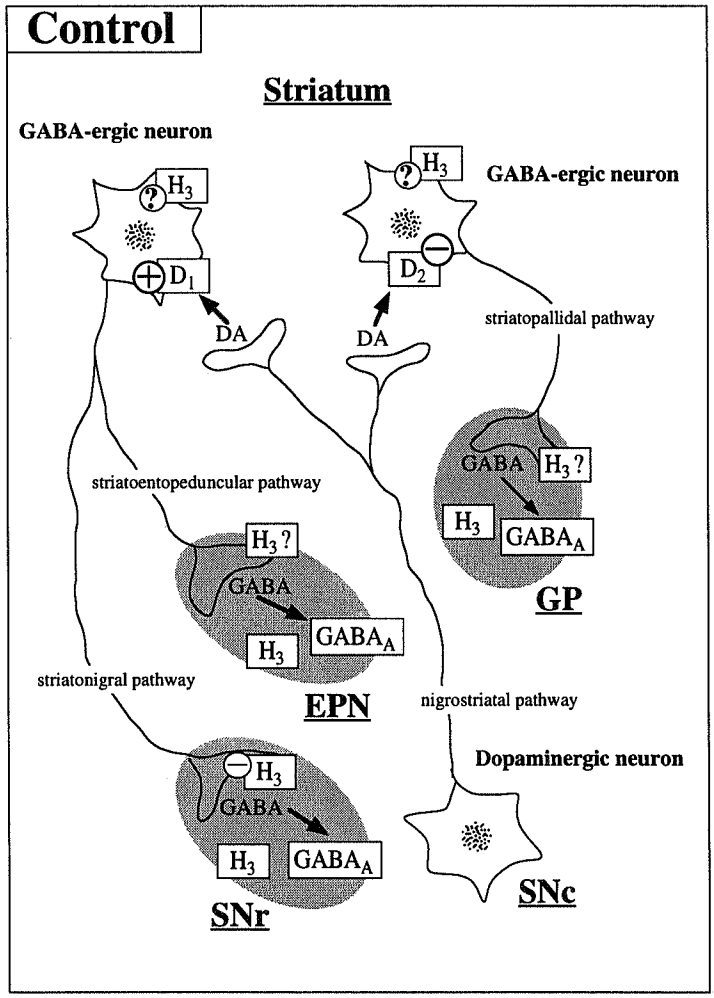
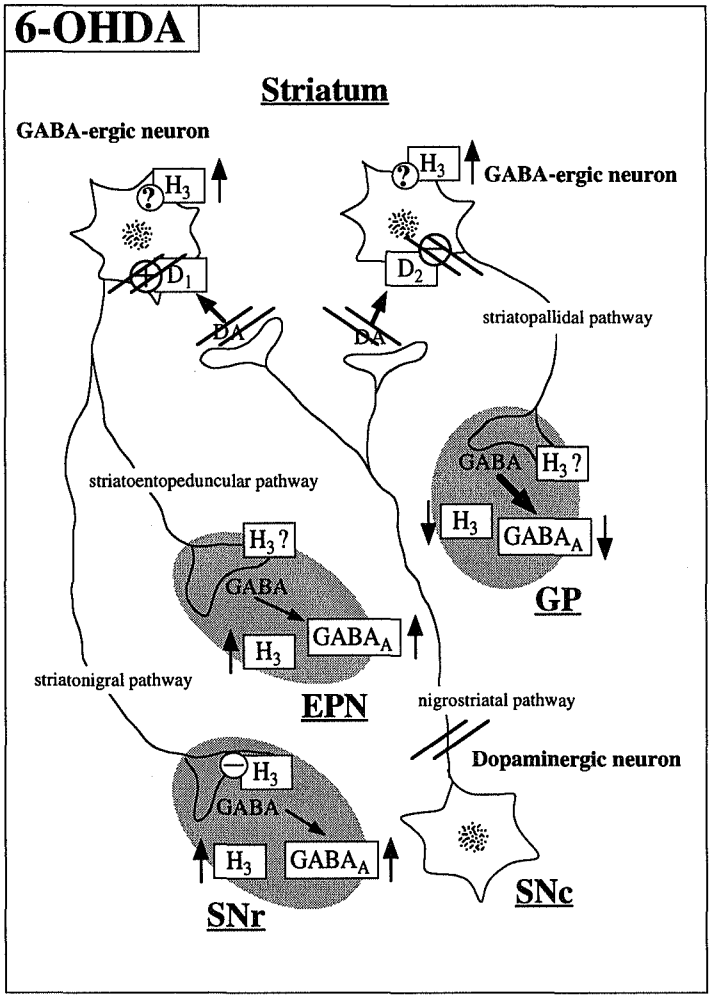


Figure 3. *Schematic representation of the effect of 6-OHDA lesions on the density of H_3-receptors in the striatum and its output centers. A hypothetic model of their localization and the overlap with $GABA_A$-receptors. Dopaminergic neurons of the nigrostriatal pathway originate from the substantia nigra, pars compacta (SNc) and terminate in the striatum. The striatal efferents to the entopeduncular nucleus (EPN), the substantia nigra pars reticulata (SNr) and to the globus pallidus (GP) use GABA as neurotransmitter (Kita & Kitai, 1988). (For references underlying the scheme: see 'discussion'). The neuronal scheme as presented is part of the basal ganglia thalamocortical circuit (Alexander & Cruther,*



1990; Gerfen et al., 1990). The striatoentopeduncular and striatonigral routes are commonly referred to as the 'direct pathway', and the striatopallidal route as the 'indirect pathway'. The latter pathway projects via the subthalamic nucleus to the substantia nigra and the entopeduncular nucleus. Stimulation and inhibition by dopamine of the direct- and of the indirect pathway, respectively, synergistically inhibits the GABA-ergic output of the SNr/EPN to the thalamus resulting in a stimulation of glutaminergic thalamocortical neurons.

Although no direct proof has been provided, several studies indicate that striatal H_3-receptors are largely of postsynaptic origin (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). Intrastriatal lesions with quinolinic acid or kainic acid, destroying postsynaptic structures (Schwarcz *et al.*, 1983), resulted in a marked loss (-60% to -70%) of striatal H_3-receptor binding sites (Cumming *et al.*, 1991; Pollard *et al.*, 1993; Ryu *et al.*, 1994). At least 85% of the striatal efferents were estimated to utilize γ-aminobutyric acid (GABA) as a neurotransmitter (Kita & Kitai, 1988). Consequently, striatal H_3-receptors may be located largely on GABA-ergic efferents.

The different effects of 6-OHDA treatment on [^{125}I]iodophenpropit binding in the SNR, the EPN and the GP may be explained neurochemically. The three areas are innervated by projection neurons of the striatum i.e. the striatonigral, the striatoentopeduncular, and the striatopallidal pathways, respectively (see Figure 3). As mentioned, these striatal efferents have been demonstrated to use GABA as their primary neurotransmitter (Kita & Kitai, 1988). The striatonigral and the striatoentopeduncular neurons contain substance P and dynorphin as co-transmitters, whereas striatopallidal neurons utilize enkephalin as co-transmitter (Gerfen *et al.*, 1990). These neurochemical differences reflect a heterogeneity of these striatal efferents. Interestingly, the altered binding of [^{125}I]iodophenpropit in the three areas mentioned above after lesions with 6-OHDA shows the same pattern as changes of $GABA_A$-receptor binding after lesions with 6-OHDA i.e. increased binding in the SNR (+32%) and in the EPN (+32%) and decreased binding in the GP (-44%) (Pan *et al.*, 1985; Gnanalingham & Robertson, 1993). These bi-directional changes of $GABA_A$-receptor binding were proposed to be related to the functional heterogeneity of the different pathways (Pan *et al.*, 1985). The striatonigral and striatoentopeduncular neurons have been demonstrated to be stimulated by striatal dopamine activating dopamine D_1-receptors (Robertson *et al.*, 1992; Pan *et al.*, 1985). Oppositely, striatopallidal neurons are inhibited by striatal dopamine through activation of dopamine D_2-receptors (Robertson *et al.*, 1992; Pan *et al.*, 1985). Thus, the increased radioligand binding in the SNR and in the EPN may be compensatory mechanisms to a reduced stimulation of both neuronal pathways due to striatal dopamine depletion. Similarly, the decreased binding in the GP may result from a reduced inhibition of striatopallidal neurons caused by striatal dopamine depletion. In line with this theory, increased binding of the histamine H_3-receptor agonist [^{3}H](R)α-methylhistamine in the SNR of 6-OHDA lesioned rats was reversed by administration of the D_1-agonist SKF38393 but was not affected by the D_2-agonist quinpirole (Ryu *et al.*, 1996). The densities of other neurotransmitter receptors like μ- and δ- opioid receptors were differently affected by 6-OHDA lesions as compared to $GABA_A$-receptors (Pan *et al.*, 1985). The similarities between the changes in histamine H_3-receptor binding compared to changes of $GABA_A$-

receptors suggest that the localization of H_3-receptors in the SNR, the EPN and the GP may partially overlap with $GABA_A$-receptors, which are predominantly localized postsynaptically (see Figure 3).

The presence of histamine H_3-receptors in the striatum and on the striatal output nuclei implies a role of central histamine in functions related to these areas. Evidence has been presented for the involvement of the central histaminergic system in locomotor activity (for review see: Onodera *et al.*, 1994). In rats, intra-accumbens administration of the H_3-receptor agonist N^{α}-methylhistamine produced hypoactivity, followed by a hyperactivity (Bristow & Bennet, 1988). Degeneration of striatal afferent and efferent pathways have been related to the etiology of different motor disorders. Experimental disruption of nigrostriatal and of striatal efferent pathways are used as model systems for Parkinson's disease (Gerfen *et al.*, 1990) and Huntington's disease (Figueredo-Cardenas *et al.*, 1994; Nicholson *et al.*, 1995), respectively. Therefore, the changed histamine H_3-receptor binding in rats after disruption of the nigrostriatal tract described in the present study, and the decreased H_3-receptor binding upon destruction of intrinsic striatal neurons might imply an involvement of histamine H_3-receptors in primate motor disorders. Also, striatal afferent and efferent pathways have been indicated to modulate epileptic seizure threshold (Turski *et al.*, 1990; Turski *et al.*, 1991). A role of the central histaminergic neuron system in the inhibition of seizures has been indicated (Yokoyama *et al.*, 1992; Yokoyama *et al.*, 1993c; Kiviranta *et al.*, 1995). Brain penetrating histamine H_1-receptor antagonists have been shown to exhibit proconvulsive activity in mice and in primates (Yokoyama *et al.*, 1993a; Yokoyama *et al.*, 1993b). Convulsions in mice were inhibited by the histamine H_3-receptor antagonists thioperamide and clobenpropit (Yokoyama *et al.*, 1993c; Yokoyama *et al.*, 1994). The precise mechanism of the anticonvulsive activity of histaminergic ligands is presently unknown. Modulation of GABA mediated neurotransmission is a target for development anti-epileptic drugs (Turski *et al.*, 1991; Palmer & Miller, 1996). With this respect, a possible co-localization of histamine H_3-receptors with $GABA_A$-receptors may be of interest. Electrophysiological and microdialysis studies have indicated a link between the histaminergic and the GABA-ergic system (Yokoyama, 1994; Okakura-Mochizuki *et al.*, 1996; Blandina 1996, personal communication). In the SNR, H_3-receptors were recently shown to inhibit the dopamine stimulated GABA release (Garcia *et al.*, 1997). However, at present data are lacking to suggest a GABA-ergic mechanism of anticonvulsant activity of the histaminergic neuronal system.

In summary, we have demonstrated an interaction between histamine H_3-receptors in the rat basal ganglia and the dopaminergic neuron system. 6-OHDA lesions of dopaminergic neurons altered histamine H_3-receptor antagonist [^{125}I]iodophenpropit binding in the

striatum and in striatal output nuclei i.e. the SNR, the EPN and the GP. The changed histamine H_3-receptor binding may be of importance in the pathology of motor disorders like Parkinson's disease and Huntington's disease and, consequently, the histamine H_3-receptor may be a new potential target in these disorders.

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- *Chapter 7* -

A quantitative and whole-body autoradiographic study of the radio-iodinated histamine H_3-receptor antagonist iodophenpropit, after intravenous administration to rats

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Abstract

The histamine H_3-receptor antagonist [^{125}I]iodophenpropit has been successfully introduced as a radioligand to study H_3-receptors in the central nervous system (CNS) *in vitro*. The present study was conducted to obtain insight into the *in vivo* distribution of iodophenpropit. [^{125}I]Iodophenpropit was intravenously injected into rats and the distribution of the radioactivity at intervals of three minutes up to four days after injection was studied by whole-body autoradiography. Using [^{131}I]-labelled iodophenpropit, the radioactivity present in different tissues after injection of the radioligand was quantified.

At three minutes after injection autoradiographic images showed a heterogeneous distribution of the radioactivity. High amounts of radioactivity were found in several peripheral tissues in which H_3-receptors have previously been identified such as lung, heart, stomach, intestine, spleen and pituitary. Only a minor fraction (of about 0.05 %) of the injected radioactivity was detected in the brain, mainly in the choroid plexus. Pre-injection of unlabelled iodophenpropit, did not substantially affect the tissue distribution of radioactivity in most tissues. After one hour considerable amounts of radioactivity were transferred to the intestine, probably via biliary excretion, indicating the onset of elimination of the compound and/or its radiolabelled metabolites via the faeces. Four days after injection the radioactivity was almost completely eliminated from all tissues except for the thyroid gland in which an accumulation was seen.

In conclusion, iodophenpropit poorly penetrates into the brain. The compound can be regarded as a potential ligand for *in vivo* pharmacological studies, being a preferentially peripherally acting H_3-receptor antagonist.

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Introduction

The histamine H_3-receptor was identified in 1983 as the autoreceptor of histaminergic neurons in the CNS (Arrang *et al.*, 1983). Besides modulation of neuronal histamine release, activation of histamine H_3-receptors has been shown to modulate the release of various other neurotransmitters such as serotonin (Fink *et al.*, 1990), noradrenaline (Schlicker *et al.*, 1989), acetylcholine (Clapham & Kilpatrick, 1992) and neuropeptides (Matsubara *et al.*, 1992). Receptor binding studies with radiolabelled H_3-receptor agonists (Pollard *et al.*, 1993; Cumming *et al.*, 1991) and antagonists (Jansen *et al.*, 1994; Ligneau *et al.*, 1994; Yanai *et al.*, 1994) showed that H_3-receptors are heterogeneously distributed in the CNS exhibiting receptor densities up to 300 fmol/mg of protein (Jansen *et al.*, 1994). Centrally acting H_3-receptor ligands have been demonstrated to modulate sleep and wakefulness, cognition, locomotion, and feeding behaviour, and were shown to affect electrically induced convulsions (for review see: Schwartz *et al.*, 1991 and Onodera *et al.*, 1994). Histamine H_3-receptors have also been functionally identified in many peripheral tissues like lung (Ichinose & Barnes, 1989; Burgaud *et al.*, 1992; Cardell & Edvinsson, 1994; Dimitriadou *et al.*, 1994), heart (Luo *et al.*, 1991; Malinowska & Schlicker, 1993; Endou *et al.*, 1994), stomach (Bado *et al.*, 1991; Coruzzi *et al.*, 1991; Bado *et al.*, 1994; Soldani *et al.*, 1994), intestine (Trzeciakowski, 1987; Hew *et al.*, 1990; Menkveld & Timmerman, 1990; Leurs *et al.*, 1991), spleen (Dimitriadou *et al.*, 1994), skin (Archer & Greaves, 1993; Ohkubo *et al.*, 1994) and perivascular tissue (Ishikawa & Sperelakis, 1987; Ea-Kim & Oudart, 1988). The wide distribution of histamine H_3-receptors suggests the involvement of this receptor in many (patho)physiological processes.

The H_3-receptor agonist (R)α-methylhistamine and the H_3-antagonist thioperamide were the first ligands used to study histamine H_3-receptors *in vitro* and *in vivo* (Arrang *et al.*, 1987). More recently, other selective H_3-receptor agonists and antagonists derived from different chemical classes have been introduced (Leurs *et al.*, 1995). In our laboratory the H_3-receptor antagonist clobenpropit has been developed, being one of the most potent histamine H_3-receptor antagonists *in vitro* known so far (Van der Goot *et al.*, 1992). Clobenpropit is a brain penetrating compound, although in rats its central effects occur at relatively high doses (5 - 15 mg/kg, s.c. or p.o.; Barnes *et al.*, 1993). [^{125}I]Iodophenpropit, a close derivative of clobenpropit, has previously been introduced as a radioligand (Menge *et al.*, 1992; Jansen *et al.*, 1992). This radiolabelled antagonist was shown to be a suitable tool for H_3-receptor studies *in vitro* (Jansen *et al.*, 1994; Leurs *et al.*, 1996). At present, no data have been provided on the *in vivo* pharmacology of iodophenpropit. Iodophenpropit and related compounds (for example its fluorinated derivatives) could have a potential application for *in vivo* imaging techniques like single

photon emission computed tomography (SPECT) and positron emission tomography (PET).
In order to get information on the *in vivo* kinetics of iodophenpropit, [^{125}I]- and [^{131}I]-labelled iodophenpropit were injected into rats. The distribution of radioactivity after injection of [^{125}I]-iodophenpropit was studied using whole-body autoradiography. [^{131}I]-Iodophenpropit was used to quantify the radioactivity present in rat tissues at different intervals after administration.

Materials and methods

Chemicals
[^{125}I]-Iodophenpropit (specific activity of 2000 Ci mmol^{-1}) and [^{131}I]-iodophenpropit (specific activity of 15000 Ci mmol^{-1}) were synthesized according to the method described by Menge *et al.* (Menge *et al.*, 1992). HPLC-Analysis showed that the [^{125}I]- and [^{131}I]-iodinated products were chemically pure and had a radiochemical purity >99%. Unlabelled iodophenpropit dihydrobromide was from laboratory stock. All other chemicals used were of analytical grade.

Whole-body autoradiographic studies
Male and female rats (160-200g, Central animal facility, Uppsala Biomedical Center) were injected intravenously with [^{125}I]iodophenpropit (9.25 MBq (250μCi)/kg), dissolved in 60 mM sodium citrate solution (injection volume: 160 to 200 μl). After the injection, rats were placed back in their home cages and received food and water *ad libitum*. The rats were killed by carbondioxide anaesthesia at intervals of 3 and 15 min, 1, 2, and 4 hours, and 1 and 4 days after injection. Whole-body autoradiography was performed according to Ullberg (Ullberg, 1954; Ullberg *et al.*, 1982). The rats were embedded in aqueous carboxymethyl cellulose and immediately frozen in hexane, cooled with dry-ice (-70°C). Sagittal cryostat sections (20 μm) were cut using a cryomicrotome (PMV 450 MP, Stockholm, Sweden) and were attached onto tape (No. 810, MMM Co., USA). The sections were freeze dried at -20°C (atmospheric pressure) for one day, and they were subsequently exposed to X-ray film (Structurix D7, Agfa-Gaevert, Germany, stored at -20°C). The films were developed after 4-6 weeks of exposure. Some sections were stained with hematoxylin-eosin for detailed anatomic examination.

Preparation of tissue samples for quantitative measurement of radioactivity
Male Wistar rats (180-200 g, Harlan C.P.B., Zeist, Netherlands) were injected with 4.63 MBq (125μCi)/kg [^{131}I]-iodophenpropit (dissolved in 60mM sodium citrate) into the

lateral tail vein (injection volume of 180 to 200 µl). At different intervals after the injection i.e. 15 and 30 min, 1 and 24 hours (four rats per interval), the animals were anaesthetized with ether and blood samples were taken by heart punction. Next, the rats were killed by cervical dislocation and the following tissues were collected: thyroid gland, brain, cerebellum, heart, lung, intestine, liver, kidneys, adrenals, pancreas, spleen, testis, prostate, skeletal muscle (hind paw), urine (24h interval) and faeces (24h interval). Rats kept for 24 hours were housed in metabolic cages, receiving food and water *ad libitum*. Tissue samples were weighed and the radioactivity present in the samples was measured by an LKB gamma counter. In a separate experiment rats were injected with unlabelled iodophenpropit (3 mg/kg, i.v., dissolved in saline, injection volume 180 to 200 µl, n=4) or saline (control group, n=4), at 5 minutes preceding injection with [^{131}I]-iodophenpropit (4.63 MBq (125µCi)/kg; procedure as described above). At 30 minutes after the injection with [^{131}I]-iodophenpropit the rats were anaesthetized with ether and were processed as described above.

Results

General autoradiographic distribution pattern

The distribution of radioactivity at different intervals after a single injection of [^{125}I]-iodophenpropit is displayed in Figure 1 (female rats series are shown). At three minutes after injections with [^{125}I]-iodophenpropit the radioactivity was heterogeneously distributed. High amounts of radioactivity were found in the kidneys, adrenals, lung, the heart muscle, liver and the upper part of the gastrointestinal tract. Also the thyroid gland, the salivary gland and the brown fat showed a high amount of radioactivity . Almost no radioactivity was detected in the CNS (Figure 1). A similar distribution was seen at fifteen minutes after injection. At two hours after the injection, a considerable amount of radioactivity had been transferred to the lower part of the intestinal tract, visible in the faeces, indicating elimination of the compound and/or its radiolabelled metabolites. At one day after injection the radioactivity was markedly reduced in most tissues (Figure 1). A relatively large amount of radioactivity was retained in the kidney medulla, the adrenal cortex, the lower part of the intestine, the brown fat, the salivary gland and the thyroid. At four days after administration of [^{125}I]-iodophenpropit, the radioactivity was eliminated almost exclusively from all tissues, except for the thyroid gland, in which a marked accumulation of radioactivity was observed. No clear differences of the autoradiographic images between male and female rats were found (data not shown).

Timecourse of the radioactivity in separate tissues

The radioactivity found in the heart was largely confined to the heart muscle, and was gradually declining after one hour following injection (Figure 1).

In the respiratory system one of the highest optical densities of radioactivity were observed in the lung (Figure 1). The radioactivity in the lung gradually decreased from two hours after injection of [^{125}I]-iodophenpropit. In the trachea the radioactivity was low.

High amounts of radioactivity were found in the digestive system. In the stomach the radioactivity was predominantly found in the glandular part part from three minutes to one hour after injection. From one hour to one day after injection of [^{125}I]iodophenpropit increasing amounts of radioactivity were transported into the intestine, indicating biliary excretion of the radioligand and/or its metabolites, which also was indicated by the spotty distribution pattern of the radioactivity in the liver at three and fifteen minutes. This pattern is characteristic for the accumulation in the bile ducts. A clear reduction of the radioactivity in the liver was observed from one hour after injection of [^{125}I]iodophenpropit. High amounts of radioactivity were also found in the salivary gland and the pancreas. The radioactivity in the digestive system was markedly reduced at one day after the injection and was almost completely excreted at four days after injection with [^{125}I]iodophenpropit. At one day after injection a relatively large amount of radioactivity was retained in the salivary gland.

A high amount of radioactivity was present in the kidneys, especially in the cortex at three and fifteen minutes after injection. At one hour, the radioactivity in the kidney cortex was reduced. A relatively large amount of radioactivity was still present in the medullary part at one day after injection. The radioactivity almost exclusively disappeared from the kidneys at four days after injection.

Almost no radioactivity was detected in the CNS. Apparently iodophenpropit has a poor ability to penetrate the blood brain barrier. The spots of radioactivity observed in the brain represent binding to the choroid plexus (Figure 1).

In the endocrine organs a relatively large amount of radioactivity was observed in the adrenal gland, the pituitary and in the thyroid gland. The radioactivity in the pituitary gradually declined from the sixty minutes interval to be eliminated at four days after the injections. In the adrenal gland, a relatively large amount of radioactivity was retained after one day. At four days after injection the radioactivity was almost completely eliminated. At one and four days after injections with [^{125}I]iodophenpropit the radioactivity in the thyroid was increased.

Of the lymphatic tissues, high amount of radioactivity was observed in the spleen. The radioactivity in the bone marrow was low, and almost no radioactivity was observed in

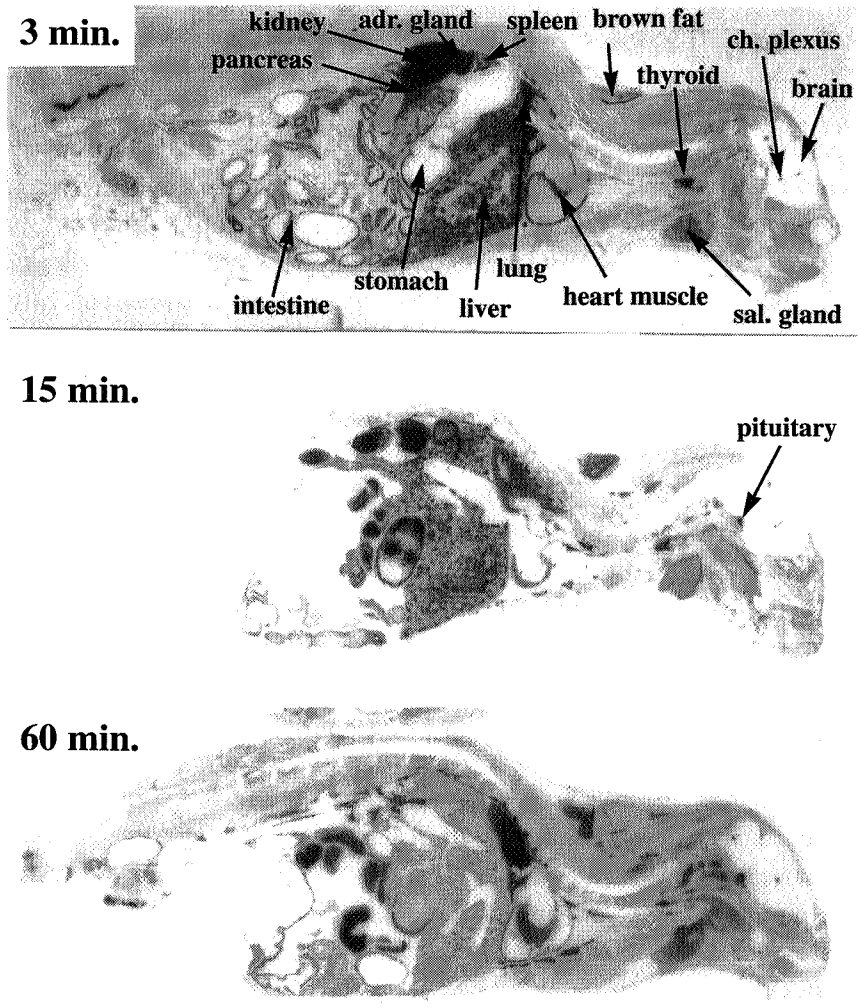

Figure 1. *Autoradiograms of rats at different intervals after intravenous injection of [^{125}I]-iodophenpropit. Rats were killed at intervals of 3 min, 15 min, 1 h, 2 h, 4 h, 1day and 4 days after injection 9.25 MBq (250μCi)/kg; see: 'materials and methods' for further details). Shown are representative autoradiograms derived from sagittal cryostat sections (20 μm) of female rats. Abbreviations: adr. gland, adrenal gland; ch. plexus, choroid plexus; sal. gland, salivary gland; thyroid, thyroid gland.*

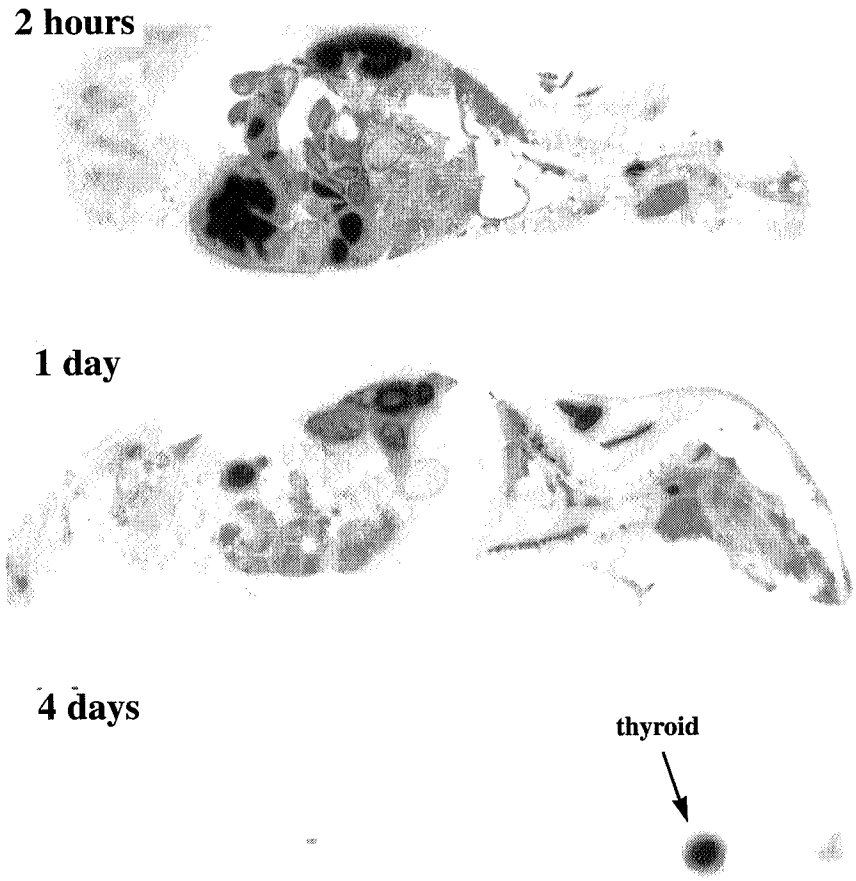

Figure 1. *(Continued).*

the thymus.
A relatively large amount of radioactivity was observed in the brown fat (see Figure 1). The radioactivity was relatively slowly eliminated from this tissue at one day after injection but almost disappeared at the four days after injection. A moderate amount of radioactivity was found in the skeletal muscles and bones.

Quantification of the radioactivity in different tissues
In a separate study the radioactivity was quantified in various male rat tissues at different intervals after injection of [^{131}I]-iodophenpropit, as shown in Table 1. The results of these experiments are in general agreement with the results of the whole-body

Table 1. *Radioactivity in rat tissues after intravenous injection of [^{131}I]-iodophenpropit.*

	% injected dose/g at different intervals after injection			
tissue	15 min	30 min	60 min	24 h
blood	0.18 ± 0.03	0.15 ± 0.01	0.15 ± 0.02	0.10 ± 0.03[a]
thyroid gland	1.22 ± 0.17	2.10 ± 0.69	1.59 ± 0.48	7.27 ± 1.85[d]
brain	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00[a]	0.01 ± 0.00[d]
cerebellum	0.05 ± 0.01	0.06 ± 0.00[a]	0.06 ± 0.02	0.03 ± 0.01[d]
heart	1.69 ± 0.23	2.11 ± 0.60	1.61 ± 0.32	0.27 ± 0.10[d]
lung	5.19 ± 0.55	5.92 ± 1.10	4.72 ± 1.14	0.88 ± 0.21[d]
intestine	1.69 ± 0.46	1.88 ± 0.71	1.92 ± 0.23	0.33 ± 0.03[d]
liver	2.33 ± 0.27	2.33 ± 0.52	1.53 ± 0.15[a]	0.20 ± 0.04[d]
kidneys	6.73 ± 0.34	6.36 ± 1.51	3.35 ± 0.28[a,b]	0.92 ± 0.13[d]
adrenals	3.82 ± 1.09	3.87 ± 0.91	3.40 ± 0.32	2.18 ± 0.27[b,c]
pancreas	1.42 ± 0.12	1.65 ± 0.35	1.41 ± 0.25	1.03 ± 0.13
spleen	3.04 ± 0.37	2.79 ± 0.86	2.21 ± 0.30[a]	0.50 ± 0.17[d]
testes	0.02 ± 0.00	0.04 ± 0.01	0.04 ± 0.01[a]	0.05 ± 0.01[a]
prostate	0.22 ± 0.01	0.34 ± 0.09	0.29 ± 0.07	0.16 ± 0.03[a,b]
muscle	0.27 ± 0.05	0.26 ± 0.05	0.23 ± 0.06	0.10 ± 0.02[a,b]
faeces	-	-	-	6.39 ± 0.40
urine	-	-	-	2.04 ± 0.23

Rats were injected with [^{131}I]-iodophenpropit (4.63 MBq (125μCi)/kg, i.v.) and tissues were collected at different intervals after the injection as described in 'materials and methods'. Values are given as the mean ± SD of four rats and were compared using a two tailed, unpaired Student's t-tests with a Bonferroni correction for multiple comparison. [a]Significantly different from 15 minute interval ($P < 0.05$), [b]significantly different from 30 minute interval ($P < 0.05$), [c]significantly different from 60 minute interval ($P < 0.05$), [d]significantly different from 15, 30 and 60 minute interval ($P < 0.05$).

autoradiographic studies. At fifteen minutes to one hour after injection the highest amounts of radioactivity were found in the lung, liver, kidneys, adrenals and the spleen (Table 1). A very low amount of radioactivity (between 0.04% - 0.05% of the total amount injected) was observed in brain tissue. From fifteen minutes to the one hour after injection no significant changes of the radioactivity in most tissues were observed. At one day after injection the radioactivity was significantly decreased in most tissues (Table 1). However, in the pancreas, the adrenals and the testes only a small reduction of the radioactivity was measured. In the thyroid gland a marked increase (about six-fold) of the radioactivity was found at one day after the injection of [^{131}I]-iodophenpropit (0.7% of the radioactivity injected).

Table 2. *The effect of injection of unlabelled iodophenpropit on the tissue distribution of the radioactivity after intravenous injection of [^{131}I]-iodophenpropit.*

	% injected dose/g, 30 minutes after injection	
tissue	saline	iodophenpropit
blood	0.40 ± 0.04	0.27 ± 0.05*
thyroid gland	2.38 ± 1.15	3.30 ± 1.18
brain	0.05 ± 0.01	0.05 ± 0.00
cerebellum	0.08 ± 0.03	0.08 ± 0.00
heart	2.26 ± 0.71	1.76 ± 0.25
lung	5.97 ± 1.75	4.14 ± 0.32
intestine	3.39 ± 1.39	2.59 ± 0.29
liver	3.08 ± 0.74	3.75 ± 0.35
kidneys	6.72 ± 0.48	7.96 ± 1.61
adrenals	5.59 ± 1.97	5.37 ± 2.82
pancreas	1.83 ± 0.61	1.89 ± 0.30
spleen	3.38 ± 0.44	2.03 ± 0.41*
testes	0.05 ± 0.01	0.05 ± 0.00
prostate	0.43 ± 0.14	0.46 ± 0.10
muscle	0.39 ± 0.15	0.42 ± 0.15

Rats were injected with [^{131}I]-iodophenpropit (4.63 MBq(125μCi)/kg, i.v.) and tissues were collected at 30 minutes after the injection as described in 'materials and methods'. Values are given as the mean ± SD of four rats. Saline or unlabelled iodophenpropit (3mg/kg, i.v.) were injected 5 minutes prior to injection of [^{131}I]-iodophenpropit. Statistical significance between the saline and the iodophenpropit injected group was considered using a two tailed, unpaired Student's t-test; *$P < 0.05$.

Pre-injection of iodophenpropit

Injection of unlabelled iodophenpropit (3 mg/kg, iv.) prior to injection of [^{131}I]-iodophenpropit did not significantly affect the distribution of the radioactivity in most tissues, as determined at thirty minutes after injection with [^{131}I]-iodophenpropit (Table 2). The effect of pre-injection of iodophenpropit on the distribution was smaller than 23% in these tissues. Injection of unlabelled iodophenpropit significantly reduced the radioactivity in the blood (-32 ± 12% of the saline injected group, n=4) and in the spleen (-40 ± 12%, n=4). A relatively large but not significant reduction and increase was found in the lung (-31 ± 5%, n=4) and in the thyroid (+39 ± 49%, n=4), respectively.

Discussion

[^{125}I]-Iodophenpropit was previously introduced as a radioligand for histamine H_3-receptor binding studies (Jansen *et al.*, 1992; Menge *et al.*, 1992). The radiolabelled H_3-receptor antagonist has been demonstrated to bind with high affinity and in a saturable and reversible manner to histamine H_3-receptors in the rat CNS (Jansen *et al.*, 1994). With its pA_2-value of 9.6, iodophenpropit is one of the most potent histamine H_3-receptor antagonists currently known. The present study was conducted to get insight in the *in vivo* potential of iodophenpropit as a radioligand. Iodophenpropit and related compounds (for example its fluorinated derivatives) could be potential tools to study the (patho) physiological role of histamine H_3-receptors e.g. using *in vivo* imaging techniques like SPECT and PET. The distribution of radioactivity was investigated after a single injection of radiolabelled iodophenpropit in rats, using whole-body autoradiography and quantitative determination.

After injection of rats with [^{125}I]- or [^{131}I]-labelled iodophenpropit, the radioactivity was heterogeneously distributed and was observed in almost all tissues. Radioactivity was present in various peripheral tissues in which histamine H_3-receptors have been functionally identified, such as lung (Ichinose & Barnes, 1989; Burgaud *et al.*, 1992; Cardell & Edvinsson, 1994; Dimitriadou *et al.*, 1994), spleen (Dimitriadou *et al.*, 1994), heart (Luo *et al.*, 1991; Malinowska & Schlicker, 1993; Endou *et al.*, 1994), stomach (Bado *et al.*, 1991; Coruzzi *et al.*, 1991; Bado *et al.*, 1994; Soldani *et al.*, 1994), intestine (Trzeciakowski, 1987; Hew *et al.*, 1990; Menkveld & Timmerman, 1990) and pituitary (Pollard *et al.*, 1993; West *et al.*, 1994). In many of these tissues the effects of histamine H_3-receptor activation are mediated by the presynaptic inhibition of neurotransmitter release. The densities of H_3-receptors in peripheral tissues are rather low (Arrang *et al.*, 1987; Korte *et al.*, 1990). Therefore, it is likely that the binding of [^{125}I]-iodophenpropit to histamine H_3-receptors in these tissues is not clearly observed in

the autoradiograms, being overruled by binding of [^{125}I]-iodophenpropit and/or of its radiolabelled metabolites to non H_3-receptor components. Consequently, it may be difficult to visualize histamine H_3-receptors in peripheral tissues *in vivo* using radiolabelled iodophenpropit. Pre-injection of unlabelled iodophenpropit did not substantially affect the distribution of the radioactivity in most tissues. However, a clear reduction was found in the spleen (-40%; $P < 0.05$) and in the lung (-31%; $P > 0.05$) after injection of unlabelled iodophenpropit. The reduced radioactivity in these tissues was not observed after pre-injection of the H_3-agonist (R)α-methylhistamine and the H_3-antagonist thioperamide, however (unpublished results). Therefore, the altered distribution of the radioactivity in these tissues after pre-injection of unlabelled iodophenpropit may not be related to the displacement of [^{125}I]iodophenpropit from H_3-receptors.

Only a small amount of radioactivity (i.e. about 0.05% of the injected dose) was detected in brain, the tissue where the highest densities of H_3-receptors are found, indicating that iodophenpropit has a poor ability to penetrate the blood brain barrier. From the autoradiographic studies it was apparent that the radioactivity found in brain tissue largely represented binding to the choroid plexus. Altogether, these results indicate that iodophenpropit may not be a suitable candidate for the *in vivo* imaging of central histamine H_3-receptors.

In the present study preliminary information is obtained with respect to the *in vivo* distribution of iodophenpropit. It may be speculated that the radioactivity distribution pattern detected after the short interval of three minutes was largely the unmodified radiolabelled compound. At one hour after injection a considerable amount of radioactivity was transferred to the lower part of the digestive system implying the onset of elimination of the compound and/or its radiolabelled metabolites. Also, at intervals from one hour the radioactivity in the kidney shifted from the cortex to the medullary part. At four days after injection the radioactivity was largely eliminated from the rat, showing that the radiolabelled iodophenpropit and/or its radioactive metabolites had almost quantitatively been eliminated. The accumulation of radioactivity in the thyroid gland indicates an involvement of deiodination in the metabolic pathway of iodophenpropit.

In conclusion, as a consequence of the poor ability of iodophenpropit to penetrate into the brain, radioiodinated iodophenpropit is not a good candidate for brain imaging of histamine H_3-receptors *in vivo*. In general, iodophenpropit can be regarded as a potential ligand for *in vivo* pharmacological studies, being a preferentially peripherally acting H_3-receptor antagonist.

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- *Chapter 8* -

The histamine H_3-receptor agonist immepip and the H_3-receptor antagonist clobenpropit modulate rat hypothalamic histamine release *in vivo*. Effects of intrahypothalamic and peripheral application

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Abstract

We investigated the effect of the new potent and selective histamine H_3-receptor agonist immepip (VUF4708) and the histamine H_3-receptor antagonist clobenpropit (VUF9153) on *in vivo* neuronal histamine release from the anterior hypothalamic area (AHy) of urethane anesthetized rats, using microdialysis. The AHy was perfused with artificial cerebrospinal fluid. Fractions were collected every 20 minutes and were assayed for histamine by an HPLC-fluorometric method.

Intrahypothalamic perfusion with the H_3-agonist immepip potently and concentration dependently reduced histamine release from the AHy. Histamine release was decreased to 75% and 35% ($P < 0.05$) of its basal level after perfusion with 1 and 10 nM immepip respectively. At concentrations of 0.1 and 1 μM immepip histamine release was below its detection limit (10 fmol/sample). Peripheral injection of immepip (5 mg/kg) caused a sustained decreased of histamine release to 50% of the basal value ($P < 0.05$). The H_3-antagonist clobenpropit potently increased histamine release after intrahypothalamic perfusion. The maximal increase of histamine release was two-fold, observed at a concentration of 10 nM clobenpropit. Histamine release was also increased upon peripheral injection of clobenpropit (5 - 15 mg/kg) to about 150% of the basal value. A more marked increase of histamine release to 250% of the basal value was observed after injection of the H_3-receptor antagonist thioperamide (5 mg/kg).

In conclusion, intrahypothalamic perfusion with the H_3-agonist immepip and the H_3-antagonist clobenpropit potently and oppositely modulated *in vivo* histamine release from the AHy. These findings support previous *in vitro* studies measuring histamine release from brain slices and synaptosomes. The decreased histamine release after peripheral injection of immepip indicates that this novel agonist readily crosses the blood brain barrier, making it a potential candidate for *in vivo* H_3-receptor studies. Hypothalamic histamine release was differentially increased upon peripheral injection of clobenpropit and of thioperamide. The possible explanation for this difference is discussed.

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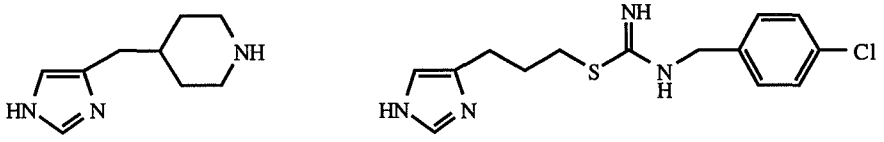
Introduction

Histamine is a well established neurotransmitter in invertebrate and vertebrate CNS including mammals (Timmerman, 1990; Onodera *et al.*, 1994; Schwartz *et al.*, 1995). Histaminergic neurons originate from the posterior hypothalamus and project in a rather diffuse way to essentially all parts of the CNS (Schwartz *et al.*, 1991; Wada *et al.*, 1991). *In vitro* techniques using brain slices and synaptosomes demonstrated that neuronal histamine release is modulated by presynaptic histamine H_3-autoreceptors (Arrang *et al.*, 1983; Van der Werf *et al.*, 1987) and by various heteroreceptors i.e. α_2-adrenoceptors (Hill & Straw, 1988), M_1-muscarinic receptors (Gulat-Marnay *et al.*, 1989), κ-opioid receptors (Gulat-Marnay *et al.*, 1990), galanin receptors (Arrang *et al.*, 1991), μ-opioid receptors (Itoh *et al.*, 1988) and nicotine receptors (Ono *et al.*, 1992). More recently, microdialysis methods were applied in order to study histamine release *in vivo* (Itoh *et al.*, 1991; Mochizuki *et al.*, 1991). It was shown that the histaminergic output from the rat hypothalamus displays a circadian rhythm (Mochizuki *et al.*, 1992). Neuronal histamine release *in vivo* was reduced by peripheral injection of α-fluoromethylhistamine, an irreversible inhibitor of the histamine synthesizing enzyme histidine decarboxylase (Mochizuki *et al.*, 1991). Microdialysis studies also demonstrated a role of H_3-autoreceptors (Mochizuki *et al.*, 1991; Itoh *et al.*, 1992; Prast *et al.*, 1994), α_2-adrenoceptors (Prast *et al.*, 1991; Laitinen *et al.*, 1995), NMDA receptors (Okakura *et al.*, 1992), interleukin-1β (Niimi *et al.*, 1994), μ-opioid receptors (Chikai *et al.*, 1994; Chikai & Saeki, 1995) and GABA receptors (Okakura-Mochizuki *et al.*, 1996) in the modulation of *in vivo* neuronal histamine release.
In vitro and *in vivo* release studies substantially contributed to the present knowledge of histaminergic neuron (patho)physiology and of the possible routes to influence neuronal histamine related effects, such as arousal, locomotor activity, learning and memory, appetite, autonomic regulation and pituitary hormone secretion (for review see: Schwartz *et al.*, 1991; Onodera *et al.*, 1994). To modify the activity of the histaminergic system, the development of brain penetrating H_3-receptor ligands is of major interest. The H_3-agonist immepip (Vollinga *et al.*, 1994) and the H_3-antagonist clobenpropit (Van der Goot *et al.*, 1992) have been described as novel potent and selective probes to study H_3-receptors *in vitro* (Figure 1). Both compounds belong to a chemical class different from the H_3-ligands which have predominantly been used for *in vivo* studies, i.e. the H_3-agonist (R)α-methylhistamine and the H_3-antagonist thioperamide (Arrang *et al.*, 1987).
In the present study we investigated the effect of intrahypothalamic and of systemic administration of immepip and of clobenpropit on *in vivo* neuronal histamine release from the anterior hypothalamus of the rat using microdialysis, to get insight in the

dynamics of hypothalamic histamine release and in the *in vivo* potential of both H_3-receptor ligands modulating neuronal histamine release.



immepip
$pD_2 = 8.0$[1)]

clobenpropit
$pA_2 = 9.9$[2)]

Figure 1. *The chemical structures of the H_3-agonist immepip and the H_3-antagonist clobenpropit. [1)]Vollinga et al., 1994; [2)]Van der Goot, et al., 1992; activities were determined by an in vitro test system measuring histamine H_3-receptor mediated relaxation of the electrically contracted guinea-pig jejunum.*

Materials and methods

Microdialysis method

The microdialysis procedure used was previously described by Mochizuki and colleagues (Mochizuki *et al.*, 1991). In brief, male Wistar rats (200-250g) were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a stereotaxic frame. A hole was drilled into the skull and subsequently, a microdialysis probe (CMA/12, Carnegie, Stockholm, Sweden) was inserted into the anterior hypothalamic area (AHy, coordinates: AP: 1.5, L: 0.5, V: 9.2 mm relative to the bregma and dural surface; see Figure 2). The AHy was continuously perfused with sterile artificial cerebrospinal fluid (artificial CSF; 2 mM sodiumphosphate buffer, 140 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM glucose, pH7.4) at a constant flow of 1 $\mu l \cdot min^{-1}$. A hundred minutes after implantation of the microdialysis probe, samples were collected every twenty minutes. Administration of immepip and clobenpropit was started after collecting the third basal fraction. The histamine content of the samples was measured as previously described, using an HPLC-fluorometric method (Yamatodani *et al.*, 1985). Briefly, 20 µl dialysate samples were mixed with 2 µl of 20% (v/v) perchloric acid and were injected into a 4 x 50 mm column packed with a cation exchanger, TSKgel SP2SW (particle size 5 µm, Tosoh). Histamine was eluted with 0.25 M KH_2PO_4 (flow: 0.6 ml/min) and derivatized by the on-line automated Shore's o-phtalaldehyde method. The detection limit for histamine was 10 fmol/sample. Neither immepip nor clobenpropit interfered with the assay of histamine.

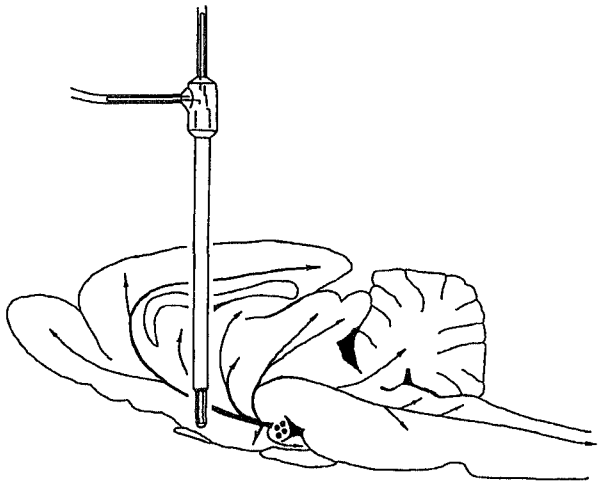


Figure 2. *Schematic representation of the microdialysis probe positioned in the anterior hypothalamic area of the rat brain.*

Drugs and chemicals

Immepip dihydrobromide (VUF4708; 4-(1*H*-imidazol-4-ylmethyl)piperidine dihydrobromide, thioperamide dimaleate and clobenpropit dihydrobromide (VUF9153) were from laboratory stock (Vrije Universiteit, Amsterdam, The Netherlands). All other chemicals used were of analytical grade.

Administration of drugs

For intrahypothalamic application immepip and clobenpropit were dissolved in artificial CSF and were infused through the microdialysis probe. When administered peripherally, drugs were dissolved in sterile sodium chloride solution (0.9% w/v) and were injected subcutaneously (volume: 1ml/kg body weight).

Statistical analysis

Histamine content of the samples after administration of drugs were compared with the histamine content of the basal fraction preceding administration of the drugs. To determine the statistical significance of the drug effects, an unpaired two-tailed Student's t-test with a Bonferroni correction for multiple comparison was used. Histamine content of the samples was considered significantly different from the basal release when P-values were smaller than 0.05.

Results

Under the experimental conditions a constant release of histamine from the anterior hypothalamic area (AHy) was observed, remaining stable from collection of the first sample up to at least five hours (Mochizuki *et al.*, 1991). The average histamine content in the dialysates was 58 ± 5 fmol/20 min (mean ± s.e.m.).

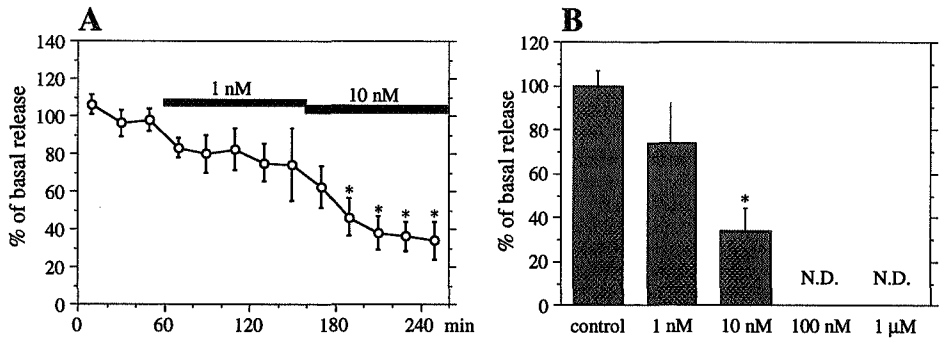


Figure 3. *The effect of intrahypothalamic perfusion with immepip on histamine release. Perfusion with immepip was started after collection of the third basal fraction, as indicated by the horizontal bars (Figure A). The values in Figure B correspond to the fourth fractions collected after perfusion with the corresponding concentrations of immepip. Values are given as the mean ± S.E.M. of three to five experiments. (*P < 0.05, compared to the third basal fraction, ND: not detected, histamine release below detection limit (10 fmol/sample).*

Effect of local and peripheral application of immepip on hypothalamic histamine release

Histamine release from the AHy was potently and concentration dependently inhibited by intrahypothalamic perfusion with the histamine H_3-receptor agonist immepip (Figure 3). At a concentration of 1 nM immepip histamine release decreased to 75% of the basal value (Figure 3A). Histamine release was significantly reduced to 35% of its basal value ($P < 0.05$) after perfusion with 10 nM immepip. At concentrations higher than 10 nM immepip the histamine release was below its detection limit (10 fmol/sample, Figure 3B). Peripheral injection of immepip at a dose of 1 mg/kg (s.c.) induced a small and transient decrease of histamine release to 70% of the basal value, 60 minutes after injection ($P < 0.05$; Figure 4). At a dose of 5 mg/kg (s.c.) immepip caused a sustained decrease of the histamine release to 50% of the basal value lasting for more than three hours.

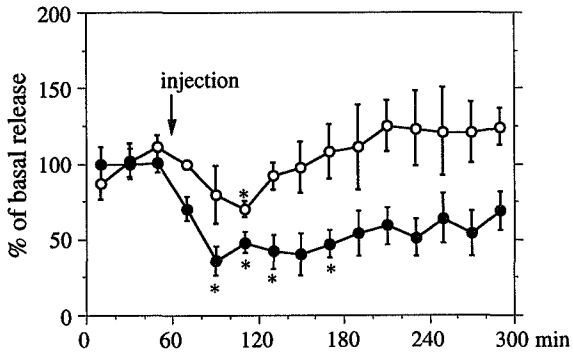


Figure 4. *The effect of sc. injection of immepip on hypothalamic histamine release. Immepip was injected after collection of the third fraction as indicated by the arrow. Values are given as the mean ± S.E.M. of four to five experiments. (*P < 0.05 compared to the third basal fraction). Symbols used: (○), 1 mg/kg; (●), 5 mg/kg immepip. Injection of saline did not affect basal histamine levels (not shown).*

Effect of local and peripheral application of clobenpropit on hypothalamic histamine release

Clobenpropit, administered through the dialysis probe, potently increased histamine release. At a clobenpropit concentration of 10 nM histamine release was significantly increased approximately two-fold (Figure 5A). Higher concentrations of clobenpropit caused no further increase of histamine release (Figure 5B). Histamine release was moderately increased upon peripheral injection of clobenpropit (5 - 15 mg/kg, s.c.), to

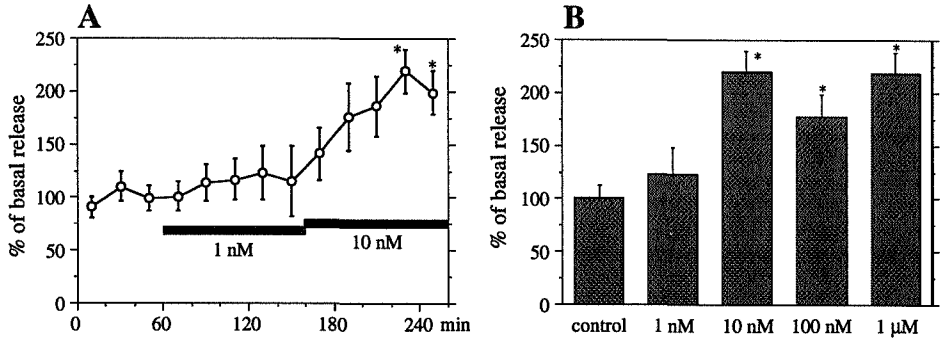


Figure 5. *The effect of intrahypothalamic perfusion with clobenpropit on histamine release. Perfusion with clobenpropit was started after collection of the third basal fraction, as indicated by the horizontal bars (Figure A). The values in Figure B correspond to the fourth fractions collected after perfusion with the corresponding concentrations of clobenpropit. Values are given as the mean ± S.E.M. of five experiments. (*P < 0.05, compared to the third basal fraction).*

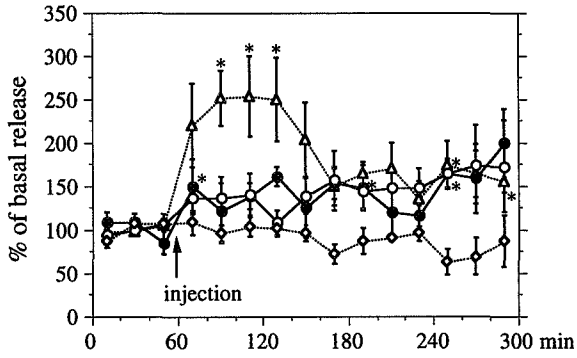


Figure 6. *The effect of sc. injection of clobenpropit and thioperamide on hypothalamic histamine release. The drugs were injected after collection of the third fraction as indicated by the arrow. Values are given as the mean ± S.E.M. of three to six experiments. (*P < 0.05, compared to the third basal fraction). Symbols used: Symbols used: (◇), 2 mg/kg; (○), 5 mg/kg; (●), 15 mg/kg clobenpropit; (△), 5 mg/kg thioperamide. Injection of saline did not affect basal histamine levels (not shown).*

about 150% of its basal value (Figure 6). In contrast, the H_3-receptor antagonist thioperamide (5 mg/kg) rapidly and markedly increased histamine release to about 250% of the basal value (Figure 6).

Discussion

The H_3-receptor agonist immepip and the H_3-antagonist clobenpropit were recently described as novel ligands exhibiting a high affinity and selectivity for H_3-receptors on the guinea-pig intestine (Vollinga *et al.*, 1994; Van der Goot *et al.*, 1992) and in rat brain (Jansen *et al.*, 1994). Immepip and clobenpropit belong to the most potent H_3-receptor ligands *in vitro*. Yet, the *in vivo* potency of both compounds has been largely unexplored. In the present study we have demonstrated that intrahypothalamic perfusion with immepip and with clobenpropit potently and oppositely modulate *in vivo* histamine release from the anterior hypothalamic area (AHy). These observations are in general agreement with *in vitro* studies describing the effects of H_3-agonists and H_3-antagonists on the depolarization induced histamine release from brain slices and synaptosomes. (Arrang *et al.*, 1983; Van der Werf *et al.*, 1987). Previous reports showed that histamine release from the AHy is almost quantitatively of neuronal origin, being sensitive to tetrodotoxin, calcium dependent and increased by electrical stimulation and infusion of potassium (Mochizuki *et al.*, 1991; Itoh *et al.*, 1992). In addition, the AHy is almost

devoid of mast cells (Olsson, 1968). As the AHy is enriched with histaminergic nerve endings, the effect of intrahypothalamic perfusion with immepip and clobenpropit is likely to be caused by their action at H_3-receptors located on these histaminergic nerve endings. The increase of histamine release by the H_3-antagonist clobenpropit may be explained by a tonic autoinhibition of histamine release from histaminergic neurons by endogenous histamine.

Intrahypothalamic perfusion with immepip induced a sustained decrease of neuronal histamine release. Previously, the effect of local perfusion with the H_3-receptor agonist (R)α-methylhistamine on neuronal histamine release has been studied, using the push-pull technique (Prast *et al.*, 1994). In the mentioned study, histamine release from the AHy was transiently decreased after perfusion with (R)α-methylhistamine. The effective concentration of (R)α-methylhistamine (10 μmol/l) to decrease histamine release largely exceeded the concentration of immepip inhibiting histamine release in our study (10 nM). It may be noted however, that in the study of Prast et al. the concentration dependency of (R)α-methylhistamine was not studied, and drugs were examined on a shorter time-scale, using a different perfusion technique (i.e. push-pull cannula). Although we could not compare the effect of intrahypothalamic perfusion with (R)α-methylhistamine in our assay, due to interference of the compound with the determination of histamine, immepip may be a more potent inhibitor of hypothalamic histamine release than (R)α-methylhistamine, when administered directly into the tissue. Accordingly, in our laboratory it was recently observed that immepip was about 20-fold more potent than (R)α-methylhistamine to inhibit the electrically stimulated [^{3}H]noradrenaline release from rat cerebral cortex slices *in vitro* (Alves-Rodrigues, personal communication). Moreover, in a recent microdialysis study it was found that the potassium stimulated acetylcholine release from rat cerebral cortex was inhibited by local administration of H_3-receptor agonists, immepip showing a 3-fold higher potency than (R)α-methylhistamine (Blandina *et al.*, 1996).

Peripheral injection of immepip (5mg/kg) induced a sustained decrease of histamine release to about 50% of its basal level. In line with this observation, peripheral injection of the same dose of (R)α-methylhistamine (5 mg/kg) has been previously shown to cause a sustained decrease of histamine release from the AHy (Itoh *et al.*, 1992). Thus, immepip has a comparable potency to (R)α-methylhistamine, when the drugs are administered peripherally. The prolonged effect of immepip on hypothalamic histamine release indicates that immepip is potentially a new pharmacological tool to study H_3-receptors *in vivo*, being able to cross the blood brain barrier. The decreased histamine release observed after peripheral injection of immepip is in accordance with its effect seen after intrahypothalamic perfusion.

At present, (R)α-methylhistamine is the most commonly used H_3-agonist for *in vivo* H_3-receptor studies. It is noteworthy though, that several studies reported non-H_3-receptor mediated effects of (R)α-methylhistamine in the peripheral tissues. *In vivo* studies on the rat cardiovascular system and the guinea-pig respiratory system revealed a histamine H_1-receptor agonistic (Hey *et al.*, 1992; Malinowska & Schlicker, 1993; Hegde *et al.*, 1994) and an α_2-adrenoceptor agonistic (Malinowska & Schlicker, 1993) activity of (R)α-methylhistamine, observed at doses required to obtain H_3-receptor mediated effects in the CNS. Also for an other brain penetrating H_3-agonist imetit, non-H_3-receptor mediated effects have been found in the rat cardiovascular system, probably resulting from a $5HT_3$-receptor agonistic activity of the compound (Coruzzi *et al.*, 1995; Leurs *et al.*, 1995). With respect to *in vivo* H_3-receptor studies immepip may therefore be an improved tool, lacking cardiovascular side effects observed with (R)α-methylhistamine and with imetit (Coruzzi *et al.*, 1995).

Histamine release was increased about two-fold after intrahypothalamic perfusion with clobenpropit. This result is comparable to local perfusion with thioperamide (Yamamoto, personal communication; Prast *et al.*, 1994). Subcutaneous injection of clobenpropit increased histamine release to about 150% of the basal value. Controversially, a marked increase of histamine release to 250% of the basal value was observed after subcutaneous injection of thioperamide (5mg/kg). It has previously been demonstrated that clobenpropit is able to penetrate into the brain. In *ex vivo* receptor binding studies the ability of clobenpropit to penetrate into the brain was about two-fold weaker than observed for thioperamide however (Barnes *et al.*, 1993; Mochizuki *et al.*, 1996). At a dose of 10 mg/kg (s.c.) clobenpropit completely abolished the increase in water consumption induced by (R)α-methylhistamine (Barnes *et al.*, 1993). Considering these data, it seems reasonable to assume that the doses of both clobenpropit and of thioperamide used in the present microdialysis study were high enough to reach a complete H_3-receptor occupancy in rat CNS.

It is not possible to give a straightforward explanation for the different effect of subcutaneous injection of clobenpropit and of thioperamide on histamine release. We would like however to make the following remarks. At first, there may be a pharmacokinetic/pharmacodynamic explanation for the different effect between the two compounds. Recently, it has has been shown that thioperamide is heterogeneously distributed in different brain areas of the rat after peripheral injection (Sakurai *et al.*, 1994). One hour after injection of a high dose of thioperamide (10 mg/kg, i.v.) relatively high tissue concentrations were found in the hypothalamus, striatum and thalamus and a relatively low concentration in the cerebral cortex. Moreover, the elimination of thioperamide was brain region dependent. Although no information on the distribution of

clobenpropit in different brain areas is available so far, a possible difference between the pharmacokinetics of thioperamide and of clobenpropit might explain the different effect of both compounds on neuronal histamine release in the hypothalamus after peripheral injection. Secondly, we suggest that the effect of peripheral injection of H_3-ligands on hypothalamic histamine release may not simply be explained by the binding of these compounds to hypothalamic H_3-autoreceptors. It has been shown that H_3-ligands are able to modulate the release of other neurotransmitters such as noradrenaline (Schlicker *et al.*, 1989), acetylcholine (Clapham & Kilpatrick, 1992; Mochizuki *et al.*, 1994) and serotonin (Fink *et al.*, 1990; Alves-Rodrigues *et al.*, 1995), by activation of H_3-receptors. Modulation of the release of neurotransmitters via H_3 heteroreceptors may in return have an effect on histamine release being potentially influenced by neurotransmitters like noradrenaline (Hill & Straw, 1988) opioid peptides (Gulat-Marnay *et al.*, 1990), acetylcholine (Gulat-Marnay *et al.*, 1989), dopamine (Prast *et al.*, 1993), glutamate (Okakura *et al.*, 1992), GABA (Okakura-Mochizuki *et al.*, 1996) and galanin (Arrang *et al.*, 1991). Therefore, the effects on histamine release of H_3-ligands injected peripherally, may be an overall result of the binding of these drugs to both H_3-autoreceptors and H_3-heteroreceptors throughout the CNS. Illustratively, it has recently been reported that the effect of the selective α_2-adrenoceptor antagonist atipamezole on hypothalamic histamine was dependent on its route of administration (Laitinen *et al.*, 1995). In this study it was found that the effects of local perfusion with α_2-adrenoceptor ligands *in vivo* generally agreed with the effects observed on noradrenaline release from brain slices *in vitro* (Laitinen *et al.*, 1995). However, local perfusion with atipamezole increased hypothalamic histamine release whereas systemic administration of the drug decreased histamine release. Therefore, it may be argued that the observations in the present study and in the study cited, cast some doubt on the usefulness of local intracerebral drug perfusion and of *in vitro* neurotransmitter release models to predict the effect of peripheral application of drugs on *in vivo* neurotransmitter release.

In conclusion, we have demonstrated that intrahypothalamic perfusion with the new H_3-agonist immepip and the H_3-antagonist clobenpropit potently and oppositely modulate *in vivo* histamine release from the AHy. These findings support previous results from *in vitro* techniques measuring histamine release from brain slices and synaptosomes. Histamine release was potently inhibited by subcutaneous injection of immepip. Hence, this H_3-agonist is potentially a new pharmacological tool to study H_3-receptors *in vivo*, being able cross the blood brain barrier. The different increase of histamine release observed upon peripheral injection of clobenpropit compared to thioperamide may be related to the complex mechanism by which systemically administered drugs influence neurotransmitter release in a specific brain area.

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- *Chapter 9* -

Summary and discussion

The H_3-receptor was first described by Arrang and colleagues in 1983, following up the identification of the histaminergic neuron system in the brain. H_3-Autoreceptors mediate the feedback inhibition of synthesis and release of neuronal histamine. Additionally, H_3-receptors are involved in modulation of the release of various other neurotransmitters in the CNS and PNS, via pre- and postsynaptic mechanisms. The role of H_3-receptors in the modulation of neurotransmitter release is briefly reviewed in Chapter 1 of this thesis.
The availability of selective ligands, both *in vitro* and *in vivo*, is of great importance to study the pharmacology of the H_3-receptor and to elucidate its physiological and pathophysiological role. Meanwhile various selective compounds are available. Progress on the synthesis of novel H_3-ligands allowed the development of different radioligands as well. Initially tritiated agonists were applied in H_3-receptor binding studies. Although radiolabelled agonists are valuable H_3-receptor binding tools, their complex binding profile urged the introduction of radiolabelled H_3-antagonists.
From 1992 up till now five different radiolabelled antagonists have been reported in literature. The first radiolabelled H_3-antagonist, [^{125}I]iodophenpropit, has been developed in our laboratory. In chapter 2 to 7 of this thesis, the validation of [^{125}I]iodophenpropit as an H_3-receptor radioligand and its consequent application to study characteristics, distribution and possible function of H_3-receptors in rodent brain is described. As an introduction to these chapters, an overview of the different H_3-receptor radioligands presented in literature so far is given in Chapter 1.

In Chapter 2 and 3, binding of [^{125}I]iodophenpropit to rat brain is described. The radioligand saturably and reversibly binds to rat cerebral cortex membranes, with an affinity closely related to its antagonistic potency. These observations, together with the analysis of competition binding curves of various histaminergic ligands and the autoradiographic examination of [^{125}I]iodophenpropit binding sites, confirmed the selective labelling by [^{125}I]iodophenpropit of sites representing the 'functional H_3-receptor'.
The binding of [^{125}I]iodophenpropit to rat cerebral cortex membranes was biphasically displaced by H_3-agonists. Based on the sensitivity of agonist competition binding curves

neurotransmitter of the basal ganglia. The striatum is enriched with dopaminergic terminals originating from the substantia nigra pars compacta (nigrostriatal system) and to less extent from the ventral tegmental area (mesolimbic system). In Chapter 6, we examined the link between rat H_3-receptors and neurotransmission related to the dopaminergic system.

Transversal brain sections of 6-hydroxydopamine (6-OHDA) treated rats were incubated with [^{125}I]iodophenpropit and the autoradiographic distribution of [^{125}I]iodophenpropit binding sites was examined. Storage phosphor autoradiography was again used to quantify the brain autoradiograms. Unilateral denervation of dopaminergic neurons altered [^{125}I]iodophenpropit binding in four different areas of the basal ganglia. In the striatum, 6-OHDA treatment produced a moderate increase of [^{125}I]iodophenpropit binding, similar to observations reported in literature using the radiolabelled agonist [^{3}H](R)α-methylhistamine (studies by Ryu *et al.*, 1994, 1996). Hence, striatal H_3-receptors may not be located on dopaminergic terminals, but rather on GABA-ergic neurons, representing the major part of the striatal efferents. These striatal efferents project to three different areas of the basal ganglia, i.e. the globus pallidus (GP), the entopeduncular nucleus (EPN) and the substantia nigra pars reticulata (SNR). Consequently, the striatal efferents are referred to as the striatopallidal, the striatoentopeduncular and the striatonigral pathway, respectively. Lesions with 6-OHDA reduced [^{125}I]iodophenpropit binding in the GP, whereas [^{125}I]iodophenpropit binding was increased in the EPN and in the SNR. These bi-directional effects of 6-OHDA treatment on [^{125}I]iodophenpropit binding can be explained neurochemically and functionally. Striatopallidal neurons are inhibited by striatal dopamine whereas striatoentopeduncular and striatonigral neurons are stimulated by striatal dopamine. Hence, the reduced [^{125}I]iodophenpropit binding in the GP and the increased [^{125}I]iodophenpropit binding in the EPN and the SNR may be compensatory mechanisms to a reduced inhibition of striatopallidal neurons and to a reduced stimulation of striatoentopeduncular and striatonigral neurons, respectively, due to striatal dopamine depletion.

Altogether, the findings described in Chapter 6 suggest that H_3-receptors in the basal ganglia play a role in neurotransmission related to the dopaminergic system.

In Chapter 7, we examined the distribution of radioactivity at different intervals after intravenous injection of radiolabelled iodophenpropit into rats. Whole-body autoradiography was used to visualize the distribution of the radioactivity. This study was conducted in order to get insight into the potential use of iodophenpropit and of its derivatives for *in vivo* imaging studies (e.g. PET and SPECT) in the CNS. At three minutes after injection of radio-iodinated iodophenpropit, the radioactivity was heterogeneously distributed and was found in many peripheral tissues in which H_3-

receptors are present. The brain penetration of iodophenpropit was low (about 0.05% of the injected dose) and the radioactivity observed in the brain was largely confined to the choroid plexus. At one hour after injection considerable amounts of radioactivity were found in the intestine indicating the onset of elimination of radiolabelled iodophenpropit and/or of its radiolabelled metabolites. The radioactivity was almost completely eliminated from all tissues at four days after injection, except for the thyroid gland.
Due to its low brain penetration iodophenpropit may not be a good candidate for brain imaging studies of H_3-receptors in vivo. Iodophenpropit can be regarded as a preferentially peripherally acting H_3-receptor antagonist.

In Chapter 8, we studied the effect of the novel H_3-receptor agonist immepip and of the H_3-antagonist clobenpropit, a close derivative of iodophenpropit, on neuronal histamine release from the anterior hypothalamus *in vivo*. Intrahypothalamic perfusion with immepip potently and concentration dependently inhibited histamine release. A decreased histamine release was also found after peripheral injection of immepip (5 mg/kg), indicating that the agonist penetrates into the brain. Immepip can be regarded as a promising tool for *in vivo* H_3-receptor studies in the CNS.
Intrahypothalamic perfusion with clobenpropit concentration dependently increased histamine release. This result indicates that neuronal histamine release is tonically inhibited *in vivo* by histamine via H_3-autoreceptors. Clobenpropit (5-15mg/kg) also increased histamine release after peripheral injection. The increase induced by clobenpropit was less pronounced than the increase found after peripheral injection of the H_3-antagonist thioperamide. Obviously, the effect of peripheral drug application on neurotransmitter release in one specific brain area is the overall result of drug effects on neurotransmission in the entire CNS. This rationale may explain the different effects of peripheral injection of clobenpropit and of thioperamide on hypothalamic histamine release.

General conclusion
This thesis demonstrates that [^{125}I]iodophenpropit selectively labels H_3-receptors in rodent CNS *in vitro*. [^{125}I]Iodophenpropit proves to be a valuable tool to obtain knowledge on characteristics, distribution and function of H_3-receptors in the CNS of rats and mice.

Samenvatting en discussie

De H_3-receptor is voor het eerst beschreven door Arrang in 1983, volgend op de identificatie van histamine als neurotransmitter in de hersenen. De H_3-autoreceptor medieert de 'feed-back remming' van de synthese en afgifte van neuronaal histamine. Tevens is de H_3-receptor betrokken bij de modulatie van de afgifte van verschillende andere transmitters in het centraal zenuwstelsel (CZS) en in het perifere zenuwstelsel, via post- en presynaptische mechanismen. In Hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van de rol van H_3-receptoren bij regulatie van de neurotransmitter-afgifte.
De beschikbaarheid van selectieve liganden, zowel *in vitro* als *in vivo*, is van groot belang bij de bestudering van de farmacologie van H_3-receptoren en bij de opheldering van de fysiologische en pathofysiologische rol van de H_3-receptor. Inmiddels zijn diverse selectieve stoffen voorhanden. De vooruitgang op het gebied van de synthese van nieuwe H_3-liganden maakte ook de ontwikkeling mogelijk van radioliganden. Aanvankelijk werden alleen H_3-agonisten toegepast in H_3-receptor bindingsstudies. Radioactieve H_3-agonisten bleken waardevol als radioliganden. Echter, het complexe bindingsprofiel van de agonisten maakte de ontwikkeling van radiogelabelde H_3-antagonisten wenselijk.
Vanaf 1992 tot op heden zijn in de literatuur vijf verschillende H_3-antagonisten als radioligand beschreven. De eerste radioactief gelabelde H_3-antagonist is [^{125}I]iodophenpropit, ontwikkeld binnen ons laboratorium. De validatie van [^{125}I]iodophenpropit als radioligand voor de H_3-receptor en de toepassing bij het bestuderen van de karakteristieken, verdeling en mogelijke functie van H_3-receptoren in ratten en muizen staat beschreven in Hoofdstuk 2 t/m 7 van dit proefschrift. Ter inleiding tot deze hoofdstukken wordt in Hoofdstuk 1 een overzicht gegeven van de verschillende radioliganden voor de H_3-receptor.

In Hoofdstuk 2 en 3 wordt de binding van [^{125}I]iodophenpropit aan rattehersenen beschreven. [^{125}I]Iodophenpropit bindt op verzadigbare en reversibele wijze aan membraanpreparaten van de cerebrale cortex van de rat. De daarbij gemeten bindings-affiniteit stemt goed overeen met de antagonistische activiteit van het radioligand. De genoemde bevindingen, samen met de analyse van competitiebindingscurven van diverse histaminerge liganden en met de waargenomen autoradiografische verdeling van [^{125}I]iodophenpropit binding, bevestigt een selectieve interactie met bindingsplaatsen die overeenkomen met de 'functionele H_3-receptor'.

De binding van [^{125}I]iodophenpropit aan cerebrale cortexmembranen van de rat werd op bifasische wijze verdrongen door H_3-agonisten. Op grond van de gevoeligheid van de agonist competitiebindingscurven voor guanine-nucleotiden, wordt geconcludeerd dat de bifasische verdringing het gevolg is van de betrokkenheid van G-eiwitten bij binding van de agonist met de receptor, en niet het gevolg van receptorheterogeniteit. Hiermee leveren de studies met [^{125}I]iodophenpropit aanvullend bewijs voor de betrokkenheid van G-eiwitten bij de H_3-receptorgemedieerde signaaltransmissie. Bifasische competitiebindingscurven werden ook gevonden voor de H_3-antagonisten burimamide en dimaprit. Mogelijk zijn deze bevindingen het gevolg van heterogeniteit van de bindingsplaatsen van [^{125}I]iodophenpropit, aangezien competitiebindingscurven van H_3-antagonisten niet werden beïnvloed door guanine-nucleotiden. Voor burimamide zijn bifasische competitiebindingscurven ook gevonden in studies waarbij gebruik werd gemaakt van H_3-agonisten als radioligand. De bifasische competitiebindingscurven zijn niet gevonden met de meer recentelijk beschreven radioactief gelabelde H_3-antagonisten. Vooralsnog wordt het bestaan van H_3-receptorsubtypen niet niet duidelijk onderbouwd door functionele studies (zie ook Hoofdstuk 1 en de discussie van Hoofdstuk 5).

In Hoofdstuk 4 wordt de binding van [^{125}I]iodophenpropit aan membranen van de hersenen van de muis bestudeerd. Muizen zijn routinematig gebruikt bij de bestudering van functionele H_3-receptor modellen. Er was echter weinig bekend over de bindingskarakteristieken van H_3-liganden ten aanzien van H_3-receptoren in het CZS van de muis, gemeten met behulp van receptorbindingsstudies. *In vivo* studies (uitgevoerd in het laboratorium van Prof. Watanabe, Sendai, Japan) toonden aan dat de H_3-antagonist clobenpropit, chemisch verwant aan iodophenpropit, een potentie heeft als anticonvulsivum in muizen, die beduidend hoger ligt dan potenties gemeten in CZS-modellen in ratten. De bovengenoemde gegevens waren aanleiding om de [^{125}I]iodophenpropit bindingsplaatsen in de hersenen van de muis nader te bestuderen. Uit deze studies is gebleken dat de affiniteiten van de meeste H_3-receptor liganden (clobenpropit inbegrepen) voor de [^{125}I]iodophenpropit bindingsplaatsen in de muizehersenen vergelijkbaar zijn met de affiniteiten gemeten in de cerebrale cortex van de rat. Deze bevindingen geven dus geen verklaring voor de *in vivo* potentieverschillen van clobenpropit tussen ratten en muizen. Competitiebindingscurven van H_3-antagonisten waren monofasisch. Daarmee werden geen aanwijzingen gevonden voor H_3-receptorheterogeniteit. Competitiebindingscurven van H_3-agonisten werden beïnvloed door guanine-nucleotiden, in overeenstemming met de interactie van de H_3-receptoren met G-eiwitten. De verdeling van H_3-receptoren in de hersenen van de muis was vergelijkbaar met de verdeling beschreven in de hersenen van rat en cavia. Echter, bij de muis werd een relatief hoge H_3-receptordichtheid gemeten in de globus pallidus. De nonspecifieke [^{125}I]iodophenpropit binding in muizehersenen

(~25%) was substantieel lager dan de nonspecifieke [^{125}I]iodophenpropit binding gemeten in de cerebrale cortex van de rat (~50%).
In hoofdstuk 5 werden de karakteristieken en distributie van [^{125}I]iodophenpropit bindingsplaatsen in het CZS van de rat bestudeerd met behulp van receptorautoradiografie. Bij de kwantificatie van de binding van [^{125}I]iodophenpropit aan verschillende hersengebieden werd gebuik gemaakt van 'storage phosphor autoradiography', een nieuwe methode waarbij de radioactief gelabelde hersencoupes worden blootgesteld aan 'storage phosphor screens', in plaats van blootstelling aan conventionele films. 'Vingerafdrukken' van de radioactief gelabelde hersencoupes werden opgeslagen in de 'storage phosphor screens'. Door het aflezen van de 'storage phosphor screens' met behulp van een 'storage phosphorimager' werden digitale weergaven van de autoradiogrammen verkregen. 'Storage phosphor autoradiography' heeft diverse voordelen ten opzichte van conventionele methoden die worden toegepast bij computerondersteunde analyse van grafische data. Een voorbeeld daarvan is de hoge mate van lineariteit over een groot gebied van radioactieve intensiteiten (beschreven in Hoofdstuk 5).
We stelden vast dat de [^{125}I]iodophenpropit bindingsplaatsen heterogeen zijn verdeeld in de hersenen van de rat. De hoogste dichtheden werden gevonden in de cerebrale cortex, het striatum en de substantia nigra. Een middelmatig lage dichtheid werd gevonden in de hypothalamus, de hippocampus en de thalamus. De [^{125}I]iodophenpropitbinding was laag in de hersenstam en in het cerebellum.
De dichtheden van de [^{125}I]iodophenpropitbindingsplaatsen gemeten in membraanpreparaten van de cerebrale rattecortex zijn substantiëel hoger dan de dichtheden van [^{3}H](R)α-methylhistaminebindingsplaatsen in hetzelfde preparaat (zie discussie van Hoofdstuk 3). Mogelijk binden radioactief gelabelde agonisten met name aan H_3-receptoren in de G-eiwit gekoppelde toestand. Als gevolg hiervan labelen de agonisten slechts een subpopulatie van de aanwezige H_3-receptoren. Met deze veronderstelling kan worden verklaard dat relatief lage H_3-receptordichtheden worden gemeten wanneer H_3-agonisten worden gebruikt als radioligand, in vergelijking met radioactief gelabelde H_3-antagonisten. Ondanks de farmacodynamische verschillen tussen H_3-agonisten en H_3-antagonisten, was het *distributiepatroon* van de [^{125}I]iodophenpropitbindingsplaatsen in de rattehersenen in algemene overeenstemming met het distributiepatroon van [^{3}H](R)α-methylhistaminebindingsplaatsen gemeten in membranen van specifieke hersengebieden van de rat (Pollard *et al.*, 1993). In de substantia nigra werd daarentegen met [^{125}I]iodophenpropit een hogere labeling gemeten dan met [^{3}H](R)α-methylhistamine. Deze constatering is mogelijk gerelateerd aan een afwijkende G-eiwitkoppeling van H_3-receptoren in de substantia nigra.

Om de [^{125}I]iodophenpropit bindingsplaatsen in de rattehersenen nader te karakteriseren, werden in verschillende hersengebieden competitiebindingscurves gemaakt van een chemisch heterogene groep H_3-antagonisten, en van de beide stereoisomeren van α-methylhistamine. 'Storage phosphor autoradiography' werd gebruikt bij de kwantificatie van de bindingscurven. De verschillende H_3-liganden maakten geen onderscheid tussen de [^{125}I]iodophenpropitbindingsplaatsen. De binding van [^{125}I]iodophenpropit in de onderzochte hersengebieden werd niet tot hetzelfde niveau verdrongen door (R)α-methylhistamine als door de H_3-antagonist thioperamide. In de meeste gebieden was de binding verdrongen door (R)α-methylhistamine 10 tot 20% lager dan de binding verdrongen door thioperamide. Grotere verschillen (tot 40%) werden gemeten in gebieden met een lagere H_3-receptordichtheid (zoals de thalamus, de hippocampus en de hypothalamus). Verschillen tussen binding verdrongen door agonisten en door antagonisten zijn ook gevonden voor andere radioactief gelabelde antagonisten. Dit fenomeen heeft belangrijke implicaties voor de definitie van de nonspecifieke binding in deze hersengebieden.

De verdeling van de H_3-receptorbindingsplaatsen is niet gelijk aan de verdeling van het histaminerge systeem. Daarom is het aannemelijk dat een groot deel van de cerebrale H_3-receptoren niet is gelegen op histaminerge neuronen. Dit geldt mogelijk in het bijzonder voor de basale ganglia, alwaar hoge H_3-receptordichtheden samengaan met een relatief geringe histaminerge innervatie. Dopamine is een belangrijke neurotransmitter in de basale ganglia. In het striatum bevinden zich veel dopaminerge zenuwuiteinden afkomstig van de substantia nigra pars compacta (nigrostriataal systeem) en, in mindere mate, dopaminerge zenuwuiteinden afkomstig van het ventrale tegmentum (mesolimbisch systeem). In Hoofdstuk 6 is het verband onderzocht tussen H_3-receptoren in de basale ganglia van de rat en neurotransmissie gerelateerd aan het dopaminerge systeem.
Transversale hersencoupes van unilateraal met 6-hydroxydopamine (6-OHDA) voorbehandelde ratten werden geïncubeerd met [^{125}I]iodophenpropit waarna de verdeling van de [^{125}I]iodophenpropitbindingsplaatsen werd bestudeerd met behulp van autoradiografie. Denervatie van dopaminerge neuronen leidde tot een veranderde [^{125}I]iodophenpropit binding in vier verschillende gebieden deeluitmakend van de basale ganglia. In het striatum van werd een geringe toename van de [^{125}I]iodophenpropit-binding gevonden, vergelijkbaar met bevindingen beschreven in studies waarbij [^{3}H](R)α-methylhistamine werd gebruikt als radioligand (Ryu *et al.*, 1994, 1996). Waarschijnlijk zijn H_3-receptoren in het striatum niet gelegen op dopaminerge zenuwuiteinden, maar voor een belangrijk deel op GABA-erge neuronen, welke het grootste deel van de striatale efferente zenuwbanen vertegenwoordigen. De striatale efferenten projecteren naar drie verschillende gebieden die deel uitmaken van de basale

ganglia te weten de globus pallidus (GP), de nucleus entopeduncularis (EPN) en de substantia nigra, pars reticulata (SNR). De striatale efferenten worden respectievelijk aangeduid als de 'striatopallidale, de striatoentopedunculaire en de striatonigrale projecties. Behandeling van ratten met 6-OHDA leidde tot een afname van de [^{125}I]iodophenpropitbinding in de GP, en tot een toename van de [^{125}I]iodophenpropitbinding in de EPN en in de SNR. Deze bi-directionele effecten kunnen worden verklaard aan de hand van neurochemische en functionele gegevens. Striatopallidale neuronen worden geremd door striataal dopamine. Daarentegen worden striatoentopedunculaire en striatonigrale neuronen gestimuleerd door striataal dopamine. De afname van de [^{125}I]iodophenpropitbinding in de GP, en de toename van de [^{125}I]iodophenpropitbinding in de EPN en in de SNR, zijn mogelijk het gevolg van compensatiemechanismen voor respectievelijk de verminderde remming van striatopallidale neuronen en de verminderde stimulatie van striatoentopedunculaire en striatonigrale neuronen, als gevolg van de depletie van dopamine in het striatum.
Samenvattend tonen de resultaten beschreven in Hoofdstuk 6 aan dat er een verband bestaat tussen H_3-receptoren in de basale ganglia van de rat en de dopaminerge neurotransmissie.

In Hoofdstuk 7 werd de verdeling van radioactiviteit onderzocht op verschillende tijdsintervallen na intraveneuze injectie van radioactief gelabeld iodophenpropit in ratten. De verdeling van de radioactiviteit werd gevisualiseerd met behulp van 'whole-body autoradiography'. Doel van de studies was inzicht te verkrijgen in de mogelijkheid om iodophenpropit en derivaten te gebruiken bij *in vivo* studies (PET of SPECT) naar de H_3-receptor in het CZS. Drie minuten na injectie van radiogelabeld iodophenpropit werd een heterogene verdeling van de radioactiviteit waargenomen. De radioactiviteit werd gemeten in verschillende perifere weefsels waarvan de aanwezigheid van H_3-receptoren is vastgesteld. De hersenpenetratie van [^{125}I]iodophenpropit was laag (ongeveer 0.05% van de geïnjecteerde dosis) en de radioactiviteit was met name waarneembaar in de plexus choroïdeus. Een uur na injectie was een aanzienlijke hoeveelheid van de radioactiviteit zichtbaar in de darm duidend op de eliminatie van radioactief gelabeld iodophenpropit en/of van zijn radioactieve metabolieten. Vier dagen na de injectie was de radioactiviteit vrijwel geheel geëlimineerd, met uitzondering van de schildklier.
Gezien de geringe hersenpenetratie van iodophenpropit is het niet waarschijnlijk dat de stof kan worden toegepast bij visualisatie van de H_3-receptor *in vivo,* na perifere toediening. Iodophenpropit kan worden beschouwd als een perifeer actieve H_3-receptor antagonist.

In Hoofdstuk 8 werd het effect onderzocht van de nieuwe H_3-receptor agonist immepip en van de H_3-antagonist clobenpropit (chemisch verwand aan iodophenpropit) op de neuronale histamineafgifte uit de anteriore hypothalamus *in vivo*. Perfusie van de hypothalamus met immepip leidde tot een concentratieafhankelijke remming van de histamineafgifte. Afname van de histamineafgifte werd ook gemeten na perifere injectie van immepip (5 mg/kg). Immepip is dus in staat om de bloed-hersenbarrière te doordringen. De stof kan worden beschouwd als een veelbelovende 'tool' om de H_3-receptor in het CZS *in vivo* te bestuderen.
Perfusie van de hypothalamus met clobenpropit leidde tot een concentratieafhankelijke toename van de histamineafgifte. Deze bevinding impliceert dat de histamineafgifte *in vivo* onder tonische invloed staat van histamine via de activering van H_3-autoreceptoren. De histamineafgifte werd ook verhoogd na perifere toediening van clobenpropit (5-15 mg/kg). De toename van de histamineafgifte was minder groot dan de toename van de histamineafgifte na perifere injectie van de H_3-antagonist thioperamide. Het effect van perifere toediening van een farmacon op de neurotransmitterafgifte in één specifiek hersengebied is de resultante van effecten van het farmacon op de neurotransmissie in het gehele CZS, hetgeen de mogelijke verklaring is voor het verschillend effect van perifere injectie van clobenpropit en van thioperamide op de histamineafgifte in de hypothalamus.

Algemene conclusie
In dit proefschrift wordt aangetoond dat [^{125}I]iodophenpropit selectief bindt aan H_3-receptoren in het CZS van ratten en muizen *in vitro*. [^{125}I]Iodophenpropit is een waardevolle 'tool' om kennis te verkrijgen omtrent de karakteristieken, de verdeling en de functie van H_3-receptoren in het CZS van ratten en muizen.

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About the author

Frank Jansen was born on August 25th 1965 in Amsterdam. After finishing secondary school (Amsterdam, June 1983) he entered the Faculty of Chemistry at the Vrije Universiteit, Amsterdam. In June 1989, he obtained his M.Sc. degree in Pharmacochemistry (Molecular Pharmacology as major subject). After fulfilling military service, he worked as a researcher at the Division of Molecular Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR), Department of Pharmacochemistry, Vrije Universiteit, Amsterdam. In january 1992 he entered the Ph.D.-programme which resulted in the present dissertation. The major part of this work concerns the pharmacological characterization of histamine H_3-receptors in the rodent central nervous system using novel ligands developed at the department. During the Ph.D.-programme he also worked periods abroad, in Japan (Department of Medical Physics, Osaka University, Osaka) and in Sweden (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala). In July, he will join the High Throughput Screening Department of Astra Draco (Lund, Sweden), as a molecular pharmacologist.

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